ERYTHROPOIESIS
IN
FETAL MOUSE LIVER

MARGARET ISABELLE F

[redacted]

[redacted]

Ex libris
universitatis
albertaensis

UNIVERSITY OF ALBERTA
QUAECUMQUE VERA

Digitized by the Internet Archive
in 2017 with funding from
University of Alberta Libraries

https://archive.org/details/fish1959

1959
11.

THE UNIVERSITY OF ALBERTA

ERYTHROPOIESIS IN FETAL MOUSE LIVER

A DISSERTATION
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

ANATOMY DEPARTMENT

by

MARGARET ISABELLE FISH, M.D.

Research Fellow of the College of
Physicians and Surgeons of Alberta
1953-54

EDMONTON, ALBERTA

APRIL, 1959.

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled ....

ERYTHROPOIESIS IN FETAL MOUSE LIVER

submitted by Margaret Isabelle Fish ....

in partial fulfilment of the requirements for the degree of Master of Science.

PROFESSOR

PROFESSOR

PROFESSOR

PROFESSOR

Date April 16 1957

ABSTRACT

A preliminary study of erythropoietic activity in embryonic liver of mice ranging in age from 9 to 15 days, through observation of sections stained with special blood stains, was carried out in order to determine an age when red blood cell proliferation was intensive. A series of tissue cultures of the liver of 11 to 15 day mouse embryos was then prepared in an attempt to observe proliferation and maturation of members of the erythropoietic blood series. Stained smears of the cut surface of embryonic liver, prepared from the embryos used in cultures, were used to complement the other studies.

ACKNOWLEDGEMENTS

The author wishes to thank Professor W. R. Salt for his guidance in the preparation of tissue cultures and for his helpful criticism and advice throughout the course of this investigation.

The assistance of Dr. H. E. Rawlinson in many ways, among them, obtaining and caring for the mice used, is gratefully acknowledged, as is Dr. J. S. Thompson's assistance in providing items of equipment.

Mr. R. Adair and Mr. R. Seward provided invaluable help in the problems of histological technique and preparation of equipment which was much appreciated.

Thanks also are extended to the staff of the university farm for providing the eggs used in the preparation of chick embryo extract for culturing.

The author would also like to thank the College of Physicians and Surgeons of Alberta for their Fellowship, and Mr. Thomas Usher for the Dorothy Jean Usher Memorial Scholarship for Medical Research, both of which made this work possible.

Special thanks are extended to Miss Therese Belanger for typing this thesis, and to the author's family for the extra time they made available to her for the preparation of the thesis.

TABLE OF CONTENTS

Page

Introduction 1

I. Where is blood formed 4
II. What cells produce blood cells 7
III. How are blood cells classified 12
IV. How do blood cells enter the circulation from their point of origin 13
V. Which blood cells move 14
VI. How do blood cells proliferate 15
VII. What is the origin and function of the megakaryocyte 16
VIII. Some special problems posed by the red blood cell series 18
  A. Is there a primitive series and then a definitive series of red blood cells in the embryo 18
  B. How is hemoglobin accumulated in the cell 20
  C. What is the fate of the red blood cell nucleus 21
  D. What is the form, metabolism and fate of the adult red blood cell 23
  E. What is the significance of the various granules occasionally demonstrable in the maturing red blood cell 25

General Aims of the present investigation 28

Materials and Methods 30
1. Animals 30
II. Fixed and Sectioned Material 35
III. In Vitro Tissue Cultures 41
IV. Tissue Smears 48
V. Vital Stains 48

Observations 50
I. Animals 50
II. Fixed and Sectioned Material 52
III. In Vitro Tissue Cultures 85
IV. Smears of Embryonic Liver 92

Discussion 101
Bibliography 121
Appendix 125

LIST OF PROCEDURES

Page

1. Custer's Zenker-Formol Fixative 37
2. Dehydration and Embedding Procedure 39
3. May-Grunwald-Giemsa Stain 40
4. Balanced Salt Solution: Tyrode 42
5. Method for Staining Whole Cultures for Best Nuclear Detail 47

LIST OF TABLES

1. Measurements of Mouse Embryos 51

LIST OF FIGURES

Page

1. Maximow Culture 44
2. Phase Culture 45
3. Cells Encountered in Embryonic Mouse Liver 55
4. 9 Day Mouse Embryo 57
5. 10 Day Mouse Embryonic Liver 61
6. Yolk Sac Vessel - 10 day 62
7. Endothelial Activity - 10 day 63
8. Normoblasts - 10 day 64
9. 11 Day Mouse Embryonic Liver 66
10. Megakaryocytes - 11 day 67
11. Oxyphilic Normoblast - 13 day 74
12. Endothelial Activity - 13 day 75
13. Nuclear Loss - 13 day 76
14. Megakaryocytes - 13 day 77
15. Embryonic Membrane Vessels - 13 day 78
16. Megakarycytes and Platelets - 14 day 81
17. 15 day 83
18. Observations of Living Cultures 89
19. Observations of Fixed and Stained Cultures 90
20. 11 day Liver Smears 93
21. 12 day Liver Smears 95
22. 13 day Liver Smears 98
23. 14 day Liver Smears 100

INTRODUCTION

For the past half century, the enigma of the formation and function of the different circulating elements of the blood has spurred scientific investigators to perfect methods of detailed study. In the evolution of their methods can be traced the progress of microscopic science. In striking contrast, as the scale of investigation has widened, the theories and generalizations have narrowed. From the first microscopic studies of dry blood smears, followed by bold speculation about the origin and function of the formed elements discovered there, much progress has been made. Ehrlich's discovery of the differential staining properties of these bodies greatly facilitated their study and reliable identification. In an attempt to study the tissues where these elements are formed, intensive microscopic examination has been carried out on sectioned material, both adult and embryonic. Stained wet imprints of hemopoietic tissues, while sacrificing tissue architecture, have allowed examination in more detail of their constituent cells. The development of in vitro tissue cultures promised a valuable means of actually observing the processes of hemopoiesis. The observation of these transparent living cells proved difficult, but with the development of the phase contrast microscope, study of unstained living material became practical. In the production of permanent records, advancement has been made from pen-and-ink interpretive sketches and camera lucida drawings, to photomicrography and cinematography. These various techniques have been applied to a variety of materials, including blood and blood-forming tissues, both embryonic and adult, of vertebrates and invertebrates. As a result of these investigations, there has arisen a great body of fact and much speculation.

Some of the more important problems posed by the study of blood have been:

1. Where is blood formed? Is blood formation an inherent capacity of certain tissues, or the result of colonization of tissues by early blood cells?

II. What cells produce blood cells? Do blood cells originate from single, specific stem cells, or are various types of cells capable of giving rise to these formed elements? If stem cells exist, does each cell type have its individual stem cell, or is there one general stem cell giving rise to multiple adult forms?

III. How are blood cells classified? How many different types of blood cells are there? Are they inherently different, or do they follow differential developmental paths due to unlike environmental stimuli?

IV. How do blood cells enter the circulation from their point of origin? Is release purely mechanical, or is it a specific response to a chemical stimulus?

V. Which blood cells move? How do they move? Is there a purpose in their movement? How can movement be utilized in the identification of cell types?

VI. How do blood cells proliferate? At what developmental level in cell differentiation is proliferation most characteristic? Is this level constant, or does it vary with different conditions? What is the mode of proliferation? Is it by mitosis, or by budding off of cytoplasm?

VII. What is the origin and function of the megakaryocyte?

Is there any relationship between platelets and megakaryocytes?

VIII. Some special problems posed by the red blood cell series are:

A. Is there a primitive series and then a definitive series of red blood cells? What would be the relation of a primitive series to the megaloblasts of pernicious anemia?

B. How is hemoglobin accumulated in the cell? What is the relation of this phenomenon to mitosis and to nuclear loss?

C. What is the fate of the red blood cell nucleus? Does it disappear by lysis, fragmentation, or extrusion? Why, despite the constancy of this phenomenon, is it so rarely observed? Is nuclear loss related in any way to the accumulation of hemoglobin?

D. What is the form, metabolism, and fate of the adult red blood cell? Does its adult form bear any relation to nuclear loss?

E. What is the significance of the various granules occasionally demonstrable in the maturing red blood cell?

The majority of the above questions have received conflicting answers from a variety of investigators. Many are still pertinent, eluding even the refinements of modern technique. The methods used in attacking these problems and the theories propounded will be considered briefly.

I. Where is blood formed?

From the microscopic study of embryo sections, much information has been elicited about the general sites of blood formation. In the embryo, blood formation starts in the wall of the yolk sac, coincident with the formation of embryonic blood islands. With the growth of the embryo, the liver and spleen soon become the chief hemopoietic organs, producing masses of blood cells. Near the time of birth, the bone marrow takes over this function, completing the transition to the adult state where the marrow is the chief source of blood cells. In addition, in the embryo, sites of blood cell production have been reported in:

a. the embryonic vascular endothelium; especially the ventral wall of the caudal portion of the dorsal aorta - (Jordan, 1916; Emmel, 1916; Maximow, 1925).

b. lymph nodes - (Bloom, 1938).

c. thymus - (Bloom, 1938).

d. the body mesenchyme - (Maximow, 1924).

e. suprarenal. Benewolenskaja (1929) quotes the observation by Freifeld and Ginsberg (1927) of erythroblasts originating from mesenchyme in the rat suprarenal.

Is blood formation an inherent capacity of these tissues? Most investigators agree that hemopoietic tissues originate from mesenchymal cells, so that any organ containing mesenchyme might function as a blood producing organ. Some state that the reticulo-endothelial tissue characteristic of liver, bone marrow, spleen, and lymph nodes provides the necessary background. Jordan (1938) drew some very interesting conclusions from a review of the available data on blood and its

development in representative vertebrates. He stated that "the primary seats of blood vessel formation (and therefore, blood cell formation, since the two are associated through the common mesenchymal ancestry of endothelium and blood cells) are determined by their topographical relation to the absorptive lining of the nutrient channel." Thus, the yolk sac, essentially a part of the primitive gastro-intestinal canal, is the initial vasculo-hemocytopoietic tissue. Likewise, he considered the spleen to be an extra-enteral portion of the submucous layer of the primitive gut (in early chordate forms, splenic tissue is incorporated in the submucous lining of the gut, as in the diffuse spleen of the lamprey). Jordan felt that the extensive sinusoidal venous system of these tissues is essential for hemoglobin elaboration in the primitive blood cells. Then, higher in the vertebrate scale, he considered that the bone marrow and liver, because of their similar sinusoidal venous system (developed originally in these sites as a "specific mechanism for optimal local nutrition") became "incidentally appropriated for blood formation." Therefore, Jordan postulated a specific cell parentage, a definite physiological environment, and adaptive environmental changes, combining to produce tissues with the inherent capacity of producing blood cells. Such a theory seems to incorporate known fact and speculation in a very reasonable manner.

By way of contrast, in 1896, Saxer (Maximow, 1924) stated that blood stem cells, formed in the wall of the yolk sac, circulated in the blood of the embryo and incidentally set up foci of hemopoiesis in organs where conditions were favorable. In recent investigations by Osogoe and Ōmura (1950), suspensions of the cellular constituents of the

bone marrow were injected into the ear veins of rabbits and the animals killed at intervals to study the fate of the cells. Accumulations of these cells were found in liver, spleen, lungs, and other organs. Although the numbers of these cells decreased rapidly and there was no trace of them after seven days, Osogoe and Ōmura have considered the fact of their transient persistence and the occurrence of mitoses as "evidence for the colonization theory with regard to the histogenesis of foci of extra-medullary hematopoiesis." However, it would seem that such transient persistence is a poor proof of colonization.

Two additional theories concerning the site of red blood cell production are in marked contrast to the above. Thomson (1914) stated that daughter nucleated red cells developed within colorless amoeboid parent cells, while Wajda (1947) asserted that the red blood cells have been seen to originate in traumatized muscle from released sarcomeres. The theories of Thomson and Wajda seem incompatible with the findings of the majority of hemotologists. Thomson's theory of the intra-cellular origin of red cells takes no realistic account of the gradual transitional stages demonstrated by all the cells of a hemopoietic tissue. Rather, it would seem to be based on isolated occurrences of phagocytosed red cells. It seems possible that the columns of red blood cells shown in Wajda's photomicrographs have originated, not from the muscle cell, but from the disrupted capillaries contained in the muscle.

Despite the very evident hemopoietic nature of organs such as the bone-marrow, spleen, and embryonic liver, their cultivation in vitro has been in general disappointing. Growth occurs, but differentiation is usually along the lines of endothelium, connective tissue, and macrophages. Some investigators, however, have reported success in the

production of blood cells.

a. Shipley (1916) observed blood cell formation in cultures of the area opaca of chick embryos from amoeboid colorless elements.

b. Maximow (1925) observed hemopoiesis in the cell clusters in the wall of the aorta of older embryos. However, he felt this to be unique as usually the only blood cells present in the culture were present at the moment of explantation.

c. Benewolenskaja (1929) described formation of red blood cells and occasional whites from hemocytoblasts in cultures of human embryonic liver.

d. Murray (1932) stated maturation of erythroblasts occurred in cultures of chick embryos of the primitive streak stage.

The over-all failure of hemopoietic tissues to show this function in vitro must be due to the unfavorable physiological environment of tissue cultures as they are presently prepared.

II. What cells produce blood cells?

The question of the origin of the cells of the blood has intrigued many an investigator. Of historical interest are such theories, listed by Isaacs (1938), as those of Weber, 1845, who theorized that red blood cells originated from liver fat globules, and Erb, 1865, who stated that the nuclei of white blood cells break into granules and take on hemoglobin staining. From the similarity of early embryonic liver cells to the intimately associated mesenchymal cells, it is easily understandable why some investigators such as Janasik, Aron and Havet in the early part of this century, postulated the origin of blood cells directly from

liver epithelium in the embryo - (Bloom, 1938). The claims of Rindfleisch and Rollet in the latter half of the 19th century that red cells originate as non-nucleated protoplasmic offspring of certain cells (Isaacs, 1938),have been reiterated rather interestingly in the contention of Emmel (1914) that the non-nucleated adult erythrocyte originates by a process of cytoplasmic constriction from the erythroblast.

In more recent years, the search for blood cell ancestors has been confined within narrower limits. It is generally accepted that the mesenchymal cell of the embryo can differentiate along several lines, i.e., a flat endothelial cell, so familiar in the lining of embryonic sinusoids; a fixed reticular cell, a stromal constituent; a basophilic round cell, a blood cell ancestor or hemocytoblast. The blood cell forming capacities of the hemocytoblast are now well accepted. However, the questions that remain disputed are:

a. Is the hemocytoblast the universal blood stem cell with distinct developmental lines producing red blood cells, white blood cells, megakaryocytes, etc.? This is the view of the monophyletic school, as professed by Danchakoff (1916); Maximow (1924); Benewolenskaja (1929); Mann and Higgins (1938); Bloom and Bartelmetz (1940); Jordan (1942); Thorell (1947); Pease (1956) and Ham (1957.

b. Are there separate, though similar, basophilic stem cells for the different series? This is the view of the polyphyletic school as professed by Stockard (1915; Sabin (1922) and Undritz (1952).

c. Does the endothelium, as found in the embryonic sinusoids,

because of its recent birth from mesenchymal tissues, retain its primitive capacities and act as the parent cell of blood cells? Bloom (1938) stated red blood cells originate from endothelium in the yolk sac. Emmel (1916), Jordon (1916), Maximow (1925) all commented on the origin of blood cells from the endothelium of a particular site, the ventral wall of the caudal end of the dorsal aorta, at a particular developmental stage in the embryo. They felt that the sprouting of aortic branches at this point and time indicated an active, relatively undifferentiated endothelium, which was capable of producing the clusters of blood cells they observed. Danchakoff (1916) postulated that endothelium as well as hemocytoblasts may give rise to red blood cells. Stockard (1915) emphatically stated there was no evidence for origin of any blood cell from endothelium. Doan, Cunningham and Sabin (1925) claimed that endothelium alone is the precursor of red blood cells, and postulated from this an extravascular, intravascular division for the differential sites of hemopoiesis; white cells in the mesenchymal tissues from basophilic stem cells and red cells in the sinusoids from the endothelium.

The diverse designations given the basophilic stem cell have caused a great deal of confusion. Thus we read of the primitive blood cells of Maximow (1924), the large lymphocyte of Maximow (1924), and the hemocytoblast of Ferrata (Bloom, 1938). Because of its basophilic

properties and superficial resemblance to the large lymphocyte, Maximow also coined the term "lymphocytoid wandering cell." This term, carrying as it does the connotation that a lymphocyte is the stem cell of the other blood cell types, has raised a great storm of controversy. This controversy can be resolved into three main stands:

(1) The term "lymphocytoid" should not be used because:
   a. the lymphocyte does not appear until late in the development of the embryo and, therefore, the suggestion that it is the stem cell is not feasible. (Sabin, 1922).
   b. Study of dry smears and imprints of hemopoietic organs prove conclusively the existence of important morphological differences between the large basophilic stem cells and adult blood and tissue lymphocytes. (Kirschbaum and Downey, 1937).

(2) An indifferent term such as hemocytoblast, cell producing blood cells, might be more appropriate, since it has not been demonstrated that lymphocytes have complete mesenchymal properties. (Bloom, 1938).

(3) From comparative studies of the invertebrate and vertebrate series, Jordan (1938) felt that the small lymphocyte and the hemocytoblast have the same multiple developmental potentialities. He arrived at this conclusion on the following grounds:
   a. In some invertebrates, the stem cell, a small element with the general characteristics of the mammalian

small lymphocyte, has been seen to differentiate into all the different types of blood cells -- leucocytes and, more rarely, erythrocytes. This would seem to be of significance in a consideration of the genesis of mammalian blood cells.

b. In the vertebrate series, he stated that the lymphocyte seems to represent an only slightly differentiated blood cell because of its spheroidal central nucleus and basophilic cytoplasm. (Undritz (1952) stated basophilia of cytoplasm is a criterion of cell youth).

c. In bird bone marrow, which contains lymphoid tissue, small lymphocytes were observed to metamorphose into hemocytoblasts and then to migrate into the capillaries to form red blood cells, while those remaining in the extravascular tissue developed into granulocytes. The differentiation into red or white blood cell lines depends upon a differential environment, most probably the higher degree of CO_2 tension in the capillaries.

It seems clear, therefore, that blood stem cells are young basophilic cells. The origin of the confusing term lymphocytoid would seem to lie in the chronology of hematological studies. That is, the earliest studies were made on adult blood smears and nomenclature of cells established at that time. Since, out of a highly varied smear cell population, the lymphocyte was the blue cell, it would be quite natural to associate these two terms, and, in later studies of hemopoietic tissues, to describe the newly discovered blue cell in terms of

the already familiar one. Such an association has led to an unfortunate confusion of terms. Certainly, in view of careful cytological studies, such as those of Kirschbaum & Downey (1937) giving minute but easily recognizable cellular and nuclear differences between the lymphocyte (whether young or adult) and the stem cell of hemopoietic tissues, the claim of Jordan (1938) for their identity, based only on the rather general similarities of basophilia, round nucleus and cell size, seems untenable.

III. How are blood cells classified?

Despite the difficulty arising from numerous systems of nomenclature, the concept of distinct lines of blood cells, distinguishable in morphology, has become well established. Thus, it is possible through detailed cytological study to place an individual cell in its proper position in these various systems. It is well accepted that there is:

a. a line of red blood cells, and

b. a line of white blood cells comprising:

   (1) a granular series with three members, neutrophils, eosinophils and basophils, recognizable by their characteristic stainable granules.

   (2) a nongranular series, including lymphocytes and monocytes.

However, the relationship of megakaryocytes and platelets to these two lines is still in dispute.

Whether or not the minute structural differences of these various formed elements are intrinsic or the expression of the action of different environmental conditions, depends almost entirely on the proof of the

existence of a single or multiple stem cells. This constitutes the core of the monophyletic-polyphyletic controversy.

Another serious problem of classification lies in the transient existence of large adult red blood cells in the embryo. That these constitute a primitive erythroblast series preceding the appearance of the definitive erythroblast series as claimed by Kirschbaum and Downey (1937), Bloom and Bartelmetz (1940), and Maximow and Bloom (1957), is not yet generally accepted. In clinical hematology, the question arises as to whether or not this primitive series is identical with the megaloblast cells of pernicious anemia.

IV. How do blood cells enter the circulation from their point of origin?

Very little specific investigation has been carried out on this question. Some theories proposed are:

a. In its maturation, the red blood cell loses its stickiness, the sinusoid opens up and the now mature cells are swept into the circulation. (Sabin, 1922).

b. Red blood cells enter the circulation by migrating through gaps in the endothelial walls of capillaries. (Jordon, 1942). This theory is supported by the electron micrographic studies of Pease (1956) showing that reticuloendothelial cells do not make a complete wall covering for sinusoids in the bone marrow.

c. Blood cells enter the capillaries from the extravascular tissue through a localized temporary solution of the capillary wall. (Isaacs, 1938).

It is evident that most of these theories are based on speculation

and that, to settle the question, more detailed observation is required.

V. Which blood cells move?

One of the exciting features of tissue culture research has been the opportunity to study the actual movements of cells. In early hematological writings, we see references to "wandering cells" (Maximow, 1924; Bloom and Bartelmetz, 1940), but it is only in the observations of investigators working on tissue cultures that we can find an accurate account of which cells move, how they move, and whether their movement can be used as a criterion for cell identification.

Various investigators have claimed the power of movement for:

a. stem cells (Jordan, 1942; De Bruyn, 1944)

b. white blood cells (Benewolenskaja, 1929; De Bruyn, 1944); Woodard and Pomerat, 1953)

c. macrocytes and megaloblasts of the primitive red cell series (Morris & Thayer, 1911).

Many tissue culture workers have commented on the migration of wandering cells away from the explant. However, De Bruyn (1944) specifically described the ameboid movement of lymphocytes and hemocytoblasts in cultures and came to the following very interesting conclusions:

1. All blood cells moving on a flat surface present the general shape of a handmirror because of the active anterior pseudopodal part.
2. When moving inside the plasma clot, all blood cells have a worm-like motion, due to the brush-heap structure of the fibrin in the plasma clot.
3. stem cells and lymphocytes are identical in their

manner of locomotion and therefore these two cell lineages cannot be separated on this basis.

4. Cells of the red blood cell series do not show independent active locomotion.

VI. How do blood cells proliferate?

Most investigators have agreed that mitotic figures can be demonstrated in the red blood cell series from the stem cell right down to the stage of the oxyphilic normoblast whose nucleus still shows some semblance of structure -- (Plum, 1947; Undritz, 1952; Maximow and Bloom, 1957).

One big question that remains is which cell stage is the truly proliferative one, renewing the supply of adult cells as the old ones are destroyed? Doan, Cunningham and Sabin (1925) stated that the erythroblasts divide extensively to produce a store of normoblasts, which are able to mature quickly through the loss of their nuclei. However, Benewolenskaja (1929), from observations on cultures of human embryonic liver, stressed the maturation of erythroblasts rather than their proliferative activity. Maximow pointed out that hemocytoblasts and erythroblasts occur in even-numbered groups indicating mitotic proliferation (Bloom, 1938). Undritz (1952) seems to have placed the proliferative level early by his statement: "Whereas rapid regeneration of the erythrocytes is almost invariably accompanied by an increase in the number of proerythroblasts, hyperplasia of the leucocytes can occur without an increased formation of blast cells ....."

Another pertinent question is: does proliferation always take place by means of mitotic division or is some other mechanism also

operative? Plum (1947), from his counts on bone marrow cultures, estimated that the number of non-nucleated cells observed, derived from normoblasts, could not be produced by the relatively slow process of mitosis. From observation of protoplasmic segregation, or gemmation, in vitro and the experimental failure of colchichine (a mitosis inhibitor) to reduce the number of mature cells formed, Plum surmised that subsequent proliferation on reaching the normoblast stage is accomplished through gemmation, and that eventually the parent cell grows old and dies as a normoblast. He estimated each normoblast gives rise to 3.92 adult erythrocytes. Descriptions of this process of gemmation to form new cells closely resemble the descriptions of budding by Cooley and Lee (1933) in the fragmentation and removal of erythrocytes from the blood stream.

Undritz (1952) made an interesting comment on mitotic division, stating that, at the time of division, besides the obvious nuclear changes, a curious mottling of the cytoplasm occurs.

It can be seen that there is no general agreement on either the stage or mode of proliferation.

VII. What is the origin and function of the megakaryocyte?

There is an extensive body of knowledge concerning this huge blood cell. Some controvery still remains regarding its origin, conflicting theories, listed by Rosenthal (1938), postulating either fusion of many cells (Di Guglielmo) or hypertrophy of a single cell (Ferrata). Most investigators, however, agree that it arises by hypertrophy from a large, basophilic cell found in the yolk sac, liver, spleen, and bone marrow. The monophyletic school identifies this latter cell as the hemocytoblast

or universal stem cell. Undritz (1952), however, representing the views of the polyphyletists, claimed that a separate stem cell can be identified for it. Little work has been done on the megakaryocyte in vitro.

From the stem cell the megakaryocyte develops by a process, unique among blood cells, termed polyploidy. Forsaking the usual mode of mitosis with division of cellular material producing separate cells of identical chromosomal and cytoplasmic makeup, the megakaryocyte undergoes a type of internal mitosis or"endomitosis" - (Undritz, 1952) within an intact nuclear membrane, producing a resultant larger nucleus with double the chromosomal content. This process is repeated until the mature cell evolves, a huge cell varying from 20 microns (Rosenthal, 1938) to 100 microns (Undritz, 1952) in diameter, with a single, huge, indented, polyploid nucleus. Polynuclear megakaryocytes also can occur both in the early blast form and under abnormal conditions (Undritz, 1952). Simultaneously, the homogeneous basophilic cytoplasm undergoes maturation changes to the granulated, oxyphilic cytoplasm of the adult cell. Typically, granulomeres, groups of reddish-violet granules, are surrounded by hyalomeres of clear cytoplasm.

Rosenthal (1938), in a historical survey, listed the following theories concerning the origin of platelets:

(1) extracorporeal origin
(2) vascular endothelial origin
(3) leucocytic origin
(4) erythrocytic origin
(5) megakaryocytic origin
(6) pluricellular origin.

That blood platelets are 1 - 2 micron fragments constricted off the cytoplasm of megakaryocytes has now been quite definitely established on the following grounds:

a. Wright in 1906 found a marked similarity in the structure and staining reaction between the granulomere and hyalomere of the blood platelet, and of the cytoplasm of the megakaryocyte pseudopodia (Ham, 1957).

b. Platelets occur only in animals with megakaryocytes. (Ham, 1957).

c. Platelets do not appear in the embryonic blood until after the appearance of megakaryocytes (Rosenthal, 1938).

d. The Feulgen reaction indicates that platelets do not have a nuclear origin (Undritz, 1952).

e. Electron microscope studies on megakaryocytes show that the cytoplasm becomes divided up into small compartments by complex invaginations of the cell membrane (Yamada, 1955).

VIII. Some special problems posed by the red blood cell series:

A. Is there a primitive series and then a definitive series of red blood cells in the embryo?

There are enough investigators propounding the existence of two distinct lines of red blood cells, -- one a transient, abortive attempt at forming circulating cells, the other establishing and maintaining the familiar red blood cell line of adult tissues, -- to warrant inspection of their claims. The main proponents are: Kirschbaum and Downey, 1937; Isaacs, 1937; Bloom and Bartelmetz, 1940; Undritz, 1952; Maximow and Bloom, 1957.

On three points there is rather consistent agreement and on three others perplexing contradiction.

It is generally agreed that the two series of red blood cells are distinguishable by:

(1) time of appearance: The primitive or megaloblast series appears early in the development of the embryo and is transient. The normoblast series appears later and persists.

(2) site of appearance: The megaloblast series issues from the yolk sac vessels while the hemopoietic organs of the embryo proper give rise to the normoblast series.

(3) distinct morphology: From both sectioned material and dry imprints of hemopoietic organs, it is apparent that the cells of the megaloblast series are larger, contain more hemoglobin, and have a more delicate chromatin network in the nucleus.

On the following questions, however, an impasse seems to have developed:

(1) stem cell: While the hemocytoblast is given as the stem cell by the monophyletic school, Undrizt (1952) stated a specific blast cell exists for each series.

(2) Are there any interconnections between the two series? The majority say no interconnections exist. Isaacs (1937, 1938), however, stated that megaloblasts occur normally in human marrow and that transition forms can

be traced between the two lines. However, experimental attempts by Jones and Smith (1950) to transform the primitive series into the definitive series met with failure.

(3) What is the relation of the primitive megaloblast to the megaloblast of pernicious anemia? Pursuing his statement that megaloblasts appear normally in the human marrow, Isaacs theorized that this cell is an integral stage of adult erythropoiesis -- a stage requiring the intrinsic factor of the stomach to mature into the normoblast. Lacking this factor, the megaloblasts do not mature normally and their large, oxyphilic descendants appear in the circulating blood. The other view, advanced first by Ehrlich in 1880, states that the megaloblast series disappears during embryonic life, and only reappears abnormally in pernicious anemia.

The intensive observations of Kirschbaum (1937) and Undritz (1952) on cellular detail, seem conclusive evidence for the existence of a primitive red blood cell series, distinct in time, origin, and morphology. The identity of this series with that of pernicious anemia must be corroborated by more extensive clinical studies.

B. How is hemoglobin accumulated in the cell?

Three interesting observations stand out in the literature on this point. Emmel (1914) remarked that hemoglobin accumulates at one pole of the cell, while the nucleus is gradually displaced to the opposite pole.

This differentiation of two distinct poles seems to be basic to his claim for eventual cytoplasmic constriction, producing the non-nucleated adult plastid.

Undritz (1952) has made an interesting correlation between mitosis and hemoglobin accumulation. He stated that during mitosis, there is a very sudden increase in hemoglobin content. The accumulation of hemoglobin during mitosis seems to be corroborated by the step-like transitions of the red cell series during its maturation.

Thorell (1947, 1953) has shown by cytochemical studies that the red blood cell series undergoes two, distinct, consecutive phases, an early growth phase, and a later maturation phase when synthesis of hemoglobin takes place.

C. What is the fate of the red blood cell nucleus?

As the cytoplasm of the basophilic normoblast metamorphoses through the polychromatic normoblast stage to the oxyphilic normoblast (by the gradual accumulation of red staining hemoglobin), a concurrent series of changes are taking place in the cell nucleus. From its original, finely detailed structure of fine, peripherally scattered dots and threads of chromatin, and well defined nucleoli, the spherical nucleus gradually changes. The nucleus shrinks and there is a progressive coarsening of the chromatin and loss of the nucleoli, until in the normoblast, the nucleus is small, dense, and for the most part, structureless. At this stage, the nucleus is lost by some process, and the mystery of this loss has engaged the curiosity of many investigators. Although the process must occur continuously, observations of the process in tissue culture are seldom reported. The following theories have been advanced as to the mode of loss:

(1) The nucleus is lost by dissolution or karyolysis. This view has been advanced by such early investigators, listed by Isaacs (1938), as Pappenheim in 1896 and Naegeli in 1931. More recently, in electron microsopic studies of embryonic blood, Muir and Kerr (1958) noted a gradual disappearance of the nucleo-cytoplasmic boundary as both cytoplasm and nucleoplasm increased in density due to hemoglobin formation in these sites.

(2) The nucleus is lost by fragmentation. Isaacs (1938) suggested that the occurrence of nuclear fragments in erythrocytes, which indicate the possibility of karyorrhexis, may be due to chromatin material which has failed to become incorporated in the nucleus after cell division.

(3) The nucleus is lost by nuclear extrusion. This theory has an interesting history. Such early investigators as Rindfleish in 1863 and Jolly in 1905, listed by Isaacs (1938), first promulgated this theory, which in more recent times has been borne out by observations of at least three tissue culture investigators, Van Herwerden in 1917, Grossman in 1923, and Rasmussen in 1933, listed by Bloom (1938). Rasmussen also stated that the extruded nuclei became shadows and suggested blood platelets.

Bloom (1938) stated that sections of hemopoietic embryonic organs demonstrate all stages in the extrusion of normoblast nuclei and their ultimate presence in phagocytes.

Emmel (1914, 1924), quarrelling with the theory of nuclear extrusion, advanced an alternative hypothesis of cytoplasmic constriction, resulting in a non-nucleated plastid of varying size and a nucleated portion with a variable rim of remaining cytoplasm.

More recently, Bessis (1955), from observation of cultures by phase microscopy, has described very vividly the actual mode of nuclear expulsion: "The cell throws out many round excrescences; one of these contains the nucleus which, after convulsive movements on the part of the cell, is expelled."

D. What is the form, metabolism and fate of the adult red blood cell?

The adult red blood cell, a flattened bi-concave disc or even a concavo-convex cup, does not owe its shape to the loss of the nucleus, but, according to the observations of Emmel (1914), assumes this definitive shape even before the loss of the nucleus.

The occasional bizarre shapes seen in normal, red blood cells are explained by the fact that the living cell is soft and easily molded by surrounding structures (Isaacs, 1938).

Under dark field illumination and ultraviolet light, no inner structure is demonstrable in the adult red blood cell (Isaacs, 1938) and it is thought that the contained material is fluid because:

1. Granules and parasites move about freely in the living cell. (Isaacs, 1938)
2. On microdissection, hemoglobin material flows out, but no fibrillae or membranes have been demonstrated. (Chambers, 1931, quoted by Isaacs, 1938, from a personal communication.)

Although Cupp (1915) stated a fine reticulum exists in both cytoplasm and nucleoplasm and this reticulum is condensed to form both the nuclear and cytoplasmic membranes, Rebuck (1948) stated that the mature red blood cell shows no stromal details.

With the loss of the nucleus, all evidence of cellular metabolism as shown by the consumption of oxygen and the giving off of CO_2, is lost (Isaacs, 1938). Despite the fact, however, that nutrition and repair must be absent in a non-nucleated cell, supposedly limiting its life span, there is little agreement in the literature on the length of life of the erythrocyte. Estimates vary from days (6-8 days judging from the reticulocyte peaks in patients with pernicious anemia treated with liver extract -- Sturgis, Isaacs, and Smith, 1928), to months (2-4 months judging from the survival of transfused blood measured by means of agglutination tests -- Wearn, Warren, and Ames, 1922; 120 days, judging from the appearance and disappearance of isotope-tagged blood cells -- Ashby, 1948). The problem is further confused by the lack of facts on pre-destruction changes in the erythrocyte.

The actual mode of destruction of the red blood cell is not known. Varying evidence has led to differing theories:

1. the cells become fragmented. (Rous and Robertson, 1917; Doan and Sabin, 1926; Cooley and Lee, 1933).
2. the cells, or fragments of them, are phagocytozed. (Doan and Sabin, 1926).
3. the cells become hemolyzed at some stage. (Isaacs, 1938).

The evidence presented would seem to suggest that all of these

mechanisms are operative in the removal of red blood cells from the circulation.

Experimental studies showing the venous blood of the spleen and marrow to contain more bilirubin than the arterial blood (Mann, Sheard, Bollman, and Baldes, 1925) seem to substantiate the generally accepted belief that these two organs (the spleen, in particular) normally function in red blood cell destruction.

E. What is the significance of the various granules occasionally demonstrable in the maturing red blood cell?

It is sometimes observed that the adult or near adult red blood cell, instead of being a homogeneous red color, contains some particulate matter in its cytoplasm. A confusing array of particles has been described in the literature. This confusion seems to result from three factors:

(1) use of different staining methods by different investigators.

(2) multiple designations for the same phenomenon.

(3) use of abnormal bloods.

A careful examination of the granules described suggests the following classification:

1. Stainable granules:

   a) Those which seem to be composed of condensed particles of the original basophilic substance of the cytoplasm of the young red cell: Four terms are commonly used to describe these granules:

      (1) basophilic stippling: a fine stippling elicited by the usual blood stains on fixed material and occasionally seen in normal bloods. (Undritz, 1952)

(2) toxic stippling: a pronounced basophilic stippling seen in diseased conditions. (Undritz, 1952).

(3) substantia granulofilamentosa: a network of granular filaments of basophilic material which can be demonstrated by supravital staining only, and is seen in the reticulocyte, the cell immediately preceding the adult red blood cell. (Undritz, 1952). (Whether or not these two above substances are identical is not clear).

(4) basophilic granules: Morris (1909) seems to use this general term for all granules not of nuclear origin.

b) Those which seem to be composed of nuclear material: Three terms are in common use:

(1) nuclear dust or chromatin dust: tiny particles considered to be remains of disintegrated nuclei in non-nucleated erythrocytes and is sometimes found in healthy persons. (Undritz, 1952)

(2) nuclear particles: single, round, circumscribed particles considered to be derived from the broken up normoblast nucleus. (Morris, 1909)

(3) Howell-Jolly bodies: dark violet, globular bodies, found in various anemias, which have been assigned varying significance: hypersmall, shrunken, normoblast nuclei (Undritz, 1952); chromosomes which have failed to be incorporated with the reconstitution

of the post-mitotic nucleus. (Isaacs, 1938; Undritz, 1952)

c) Those which are of disputed origin, such as:

(1) Cabot rings: basophilic rings seen in erythrocytes in severe anemias. There is no evidence to date that these are of nuclear material. (Isaacs, 1938)

(2) Heinz-Ehrlich bodies: highly refractile bodies, requiring special stains, found in the red blood cells of poisoned lab animals and in some human blood diseases. Their origin and significance is highly disputed. (Isaacs, 1938)

2. A non-stainable refractive granule: which resists staining by supravital or Romanowsky stains. It is a single, highly refractile granule seen in nucleated and non-nucleated cells and free in the plasma, and is characterized by its dancing motion within the cell. These cells represent a normal stage in red blood cell maturation. (Isaacs, 1938)

Careful differential staining seems to substantiate the validity of the above general classification. Thus, specific nuclear stains -- especially the Feulgen reaction (specific for chromatin material) -- establish the nuclear genesis of particles such as Howell-Jolly bodies (Undritz, 1952). Also, the demonstration in a single cell of both nuclear particles and basophilic granules, which react in a strikingly dissimilar manner to a specific stain (Pappenheim's methyl green-pyronin mixture), is unmistakable proof of the existence of at least two types

of particulate matter (Morris, 1909). Morris, however, has suggested that both may be of nuclear origin, the differential staining being due to the absence of a nuclear membrane in the basophilic granules.

Thus, while a more than tentative identity has been established for these two types of particles, the nature of the refractile granule described by Isaacs is still undetermined.

In the different areas of investigation outlined above, most of the facts have come from observations on fixed and stained material. Tissue culture research on hemopoietic organs has given tentative answers to the questions of the stem cell, cell motility, cell proliferation, and the fate of the red blood cell nucleus.

The use of supra vital stains in attempts at hematological cell identification in vitro may greatly facilitate tissue culture investigation. However, the results obtained with this technique to date have been disappointing.

Further progress in hemopoietic investigations will probably come in the perfection of phase microscopic and electron microscopic techniques.

General aims of the present investigation:

The aim of the present investigation is to study hemopoiesis and, in particular, erythropoiesis, in the embryonic liver of the mouse.

The plan of investigation is as follows:

1. a preliminary study of sectioned, stained embryos of different ages, to determine an age when red blood cell proliferation in the liver is most marked.

2. observation of tissue cultures of embryonic liver, of a good proliferative age, by phase microscopy, in an attempt:
   a. to identify a member of the red blood cell series, preferably early in its development.
   b. to observe the maturation of this cell, its mode of proliferation, its motility, and the loss of its nucleus, in the hope of shedding some light on these problems.

MATERIALS AND METHODS

General Aim: The employment of those materials and methods ensuring excellence of results, combined with reliability and reproducibility.

The discussion of materials and methods used falls naturally into the following five categories:

I. Animals
II. Fixed and Sectioned Material
III. In Vitro Tissue Cultures
IV. Tissue Smears
V. Vital Staining

I. Animals:-

For the study of hemopoiesis in embryonic liver, the mouse was chosen as a suitable and desirable laboratory animal for the following reasons:

a. It is a small mammal, easily cared for, presenting a blood picture very close to that of the human.

b. The short gestation period is admirably suited to the procuring of adequate material for study.

c. The ready availability of established and recognized pure strains tends to ensure reproducible results by separate investigators.

Two main problems were encountered in the use of mice. Initially,

it was considered desirable to inbreed an established and recognized strain of mice in order to ensure reproducible results. However, difficulty was experienced here in obtaining litters, and when comparing results obtained from breeding cages of inbred stock on the one hand and hybrid stock on the other, the influence of hybrid vigor was quite apparent. Snell (1941) commented on the production of considerably larger litters by many hybrid animals. In a practical endeavour, therefore, to obtain sufficient material in the limited time available, crosses between two established strains of mice were used throughout. The strains used were:

1. A (L.C. Strong of Yale).
2. C57 Bl (Bittner).

The second problem concerned the establishment of a method of obtaining sufficient numbers of embryos of reliably determinable ages. Such a routine entailed:

A. facilitating the production of pregnancies.
B. detecting evidence of copulation.
C. timing the pregnancy as accurately as possible.
D. establishing a check on the estimated age.

A. facilitating the production of pregnancies:

Two methods were tried:

1. The multiple chance method, wherein breeding cages were set up with two or three males (of one strain) and two or three females (of the other strain). These were set up in the evening because of the generally accepted fact that breeding in mice usually occurs

between the hours of ten p.m. and one a.m. (Snell, 1941).

2. The vaginal smear method, wherein time of estrus was ascertained by microscopic examination of a smear of the vaginal secretions, and a breeding cage set up accordingly.

Three distinct difficulties were apparent:

a) The exact determination of the microscopic picture of estrus: Snell (1941) stated that there is a striking strain difference.

b) The short duration of estrus, or heat, which is thought to last approximately twelve hours in mice. (Snell, 1941)

c) The inconvenient time of onset of estrus, which is thought to commence at night, most commonly between 10 p.m. and 1 a.m. (Snell, 1941)

In following the vaginal smear method, it was necessary to take smears from several mice, and accurately determine proestrus by microscopic examination, so that breeding cages could be set up that evening. This entailed time consuming procedures in obtaining the smears and appropriately marking the females (so that a check could be made on results). The results, in terms of reliably predicted pregnancies, did not seem to justify the time entailed.

However, by setting up breeding cages as in method 1 it was possible to obtain the necessary matings, as evidenced by the presence of vaginal plugs on subsequent mornings.

B. detecting evidence of copulation:

Evidence of copulation in the mouse is the existence in the female of a vaginal plug of white, gelatinous material easily detectable at the introitus. This plug is a mixture of the secretions of the vesicular and coagulating glands of the male (Snell, 1941) and persists for eighteen to twenty-four hours in the vagina. Females showing vaginal plugs were given distinctive markings and transferred to an embryo box. From the index card on this box, listing mouse color, marking, and date of vaginal plug discovery, it was relatively simple to choose a mouse at the desired period of gestation.

C. timing the pregnancy as accurately as possible:

A consistent method was necessary for timing embryos which would minimize and approximately standardize the discrepancy between actual and estimated age. Estrus commonly commences in the evening and is generally considered to persist twelve hours. Ovulation takes place spontaneously during estrus whether or not mating occurs, and ordinarily follows the onset of estrus within, roughly, two hours (Snell, 1941). Ova remain viable several hours, and sperm about six hours in the uterus and tubes. By setting up breeding cages around ten o'clock in the evening, and inspecting for vaginal plugs and performing dissections around ten in the morning it was possible to determine embryonic age to within twelve hours. However, in point of fact, the possibility of the extremes of this range occurring is quite small, and it is much more probable that copulation and ovulation take place within several hours of each other and in the early hours of the morning. Snell (1941) stated that most matings occur between ten and one a.m. Thus, there is probably

a difference in age of one to three hours for embryos timed in this manner. This developmental range could account for divergences in size of embryos of separate litters, but of apparently equal age. The variable developmental period prior to ten a.m. was ignored and embryonic age was calculated from approximately ten a.m. of the day of discovery of the vaginal plug.

D. establishing a check on the estimated age:

Three common methods of calculating embryonic age in mice were available:

1. estimation from vaginal plugs.
2. measurement of crown-rump length of the embryo.
3. recognition of certain external developmental features of the embryo.

Gruneberg (1943) commented on the above three methods. His criticism of the crown-rump measurement method is based on three points:

(a) there is an overlap between age and measurement, so that no one measurement is typical of any one age.

(b) the gelatinous condition of young embryos of nine to eleven days causes them to spring open when removed from their membranous sacs, precluding reliable measurements.

(c) the presence of retarded fetuses distorts the average litter measurement.

Gruneberg stated that it is possible to estimate embryonic age within twenty-four hours by identifying certain distinctive, external features characteristic of each age. It was felt, however, that a twelve hour

leeway in vaginal plug timing, together with the approximate check of crown-rump measurement, gave a reliable and consistent method of timing which was simple, rapid, and relatively free from subjective error, and this was the method adopted.

A vernier caliper was used for all measurements and the average embryonic size determined for most litters.

II. Fixed and Sectioned Material:

In the study of hemopoiesis the detail required is cytological, so that an attempt was made:

a. to establish methods of fixing and dehydrating delicate embryonic tissue so that maximum cellular detail would be preserved.

b. to determine a stain which would clearly differentiate the individual characteristics of the developing red blood cell.

Nine to fifteen day mouse embryos were used. The pregnant female mouse was killed by decapitation with scissors and immediately dissected; the embryos were dissected free of their membranes and fixed immediately. The entire embryo, or, in the case of larger embryos, either the trunk or the dissected liver, was fixed.

Several fixatives were tried:

1. 4% neutral saline formaldehyde
   (formalin; CaCl2: water; CaCO3)
   at pH 7.2

2. Custer's Zenker-Formol: (see Proc. 1)
   (Zenker's; neutral formol) (Lee, 1950)

3. Maximow's Zenker-Formol:
   (Zenker's; formol; osmic acid)

4. Picric Formol - (Bouin's solution)
   (Picric acid; formol) pH 7.

Custer's Zenker-Formol (Proc. 1) was found to give the best results. Initially, tissues were fixed at room temperature, while in later experiments incubation at 37 deg. C. for a shorter period was tried. This latter method proved to be inferior.

CUSTER'S ZENKER-FORMOL FIXATIVE - (LEE, 1950)

9 parts Zenker solution:- freshly prepared
- (without acetic acid)

1 part neutral formol: added at time of fixation.

Neutralization of formol (Baker's method - Lee, 1950)

Formalin (40% formaldehyde) buffered with $CaCO_3/CaCl_2$ and brought up to pH 7 with NaOH and HCl.

Tissues were left in fixative overnight (or approximately 8-10 hours) and then washed in running, cold water, usually overnight.

PROCEDURE 1.

After some experimentation, a dehydration and embedding schedule for delicate embryos was worked out. By stressing gradual changes in reagents, and short exposure time, shrinkage of cells was minimized. The procedure followed is outlined in Procedure 2.

Tissues were embedded in Fisher Tissue Mat (M.P.-58 deg.C) and the paraffin blocks sectioned at 6 microns with a rotary microtome. In the case of entire embryos, representative sections from the paraffin ribbon were mounted and stained with alum haematoxylin and eosin to permit identification of those sections containing the liver. Ten to twelve slides of liver tissue were then prepared from each embryo.

STAINING:

Several stains were tried, i.e., alum haematoxylin and eosin; Jenner; Giemsa-Jenner; Maximow; Wright; and May-Grunwald-Giemsa. The May-Grunwald-Giemsa stain was found to give the best differentiation. Some difficulty was encountered in adjusting the stain pH. The alkaline reaction of the tissues, due to the buffered formol used in tissue fixation, interfered with proper staining, the tissues staining too blue. By lowering the pH of the Giemsa stain mixture to between 4 and 5, much better differential staining was obtained. Staining times were also adjusted to give optimal results. See Procedure 3. for staining procedure.

DEHYDRATION AND EMBEDDING PROCEDURE

20% ethyl alcohol	1 hour
40% " "	1 "
70% " "	1 "
85% " "	$\frac{1}{2}$ "
95% " "	$\frac{1}{2}$ "
95% alcohol and cedarwood oil - 50/50 (tissue is left until it migrates from the alcohol layer to the oil)	
cedarwood oil 1	1 "
" " 2	1 "
saturated solution of paraffin in $CHCl_3$, gradually brought up from room temperature to 58 deg. C. in a water bath -	1 "

In Oven - at approx. 56-58 deg. C.

paraffin and $CHCl_3$ - 50/50	1 "
paraffin	1 "

(Tissue mat may be substituted for paraffin in the above procedure)

PROCEDURE 2.

MAY-GRUNWALD-GIEMSA STAIN

		sometimes
Xylol 1	3 minutes	
" 2	3 "	
Absolute ethyl alcohol	3 "	
Iodine in 95% ethyl alcohol	5 "	
Absolute ethy alcohol	5 "	
Absolute methyl alcohol 1	3 "	
" " " 2	3 "	
May-Grunwald stain -	4 "	2 minutes
dilute Giemsa stain (1:10-1:15 in distilled water)	16 "	8 "
Glass-distilled water		
Acetone 1	momentarily	
" 2		
Acetone-Xylol - 50/50		
Xylol 1	3 "	
" 2	3 "	

Picolyte and coverslip and put in drying oven (2-3 days)

PROCEDURE 3.

III. In Vitro Tissue Cultures:

In order to study hemopoiesis as it might occur in the living embryo, an attempt was made to prepare aseptically in vitro liver cultures as closely simulating the "**milieu interne**" as possible, in the hope that the liver tissue explant would show growth and differentiation of cells.

Culturing was carried out in a room designed to keep dust at a minimum. A newly decapitated mouse, the fur of which was well saturated with seventy percent alcohol, was dissected with instruments sterilized by immersion in 70% alcohol followed by flaming, and the pregnant uterus removed to a sterile Petri dish containing sterile, Tyrode's balanced salt solution (Procedure 4). Separation of the embryos from their placentas and sacs was accomplished in this Petri dish. The low power of a binocular microscope, equipped with a glass dissecting shield, was used while dissecting out the liver, which was then cut into pieces approximately one millimeter square. These were placed immediately in balanced salt solution in another Petri dish and set in the incubator until required for culturing.

To facilitate sterilization of equipment and rapid routine preparation for culturing, a culturing kit was constructed (after the pattern devised and employed in the Strangeways Lab., Cambridge, England). This consisted of an approximately square, wire-mesh container with asbestos bottom, packed with the requisite glassware, pipettes, knives, scissors, etc., all in appropriate, separate containers.

BALANCED SALT SOLUTION: TYRODE

1000 cc. Tyrode		500 cc.
NaCl	8 grams	4.0 grams
KCl	0.2 "	00.1 "
CaCl2	0.2 "	0.1 "
MgCl2	0.1 "	0.05 "
NaH2PO4	0.05 "	0.025 "
NaHCO3	1.0 "	0.5 "
Glucose	1.0 "	0.5 "

Double glass distilled water to 1,000 cc.

Adjust with N/10 HCl to pH 7.4-7.8, using Phenol Red.

Use Seitz filter (standard pad).

PROCEDURE 4.

Two distinct culture techniques were employed:

a. standard Maximow double coverslip cultures.

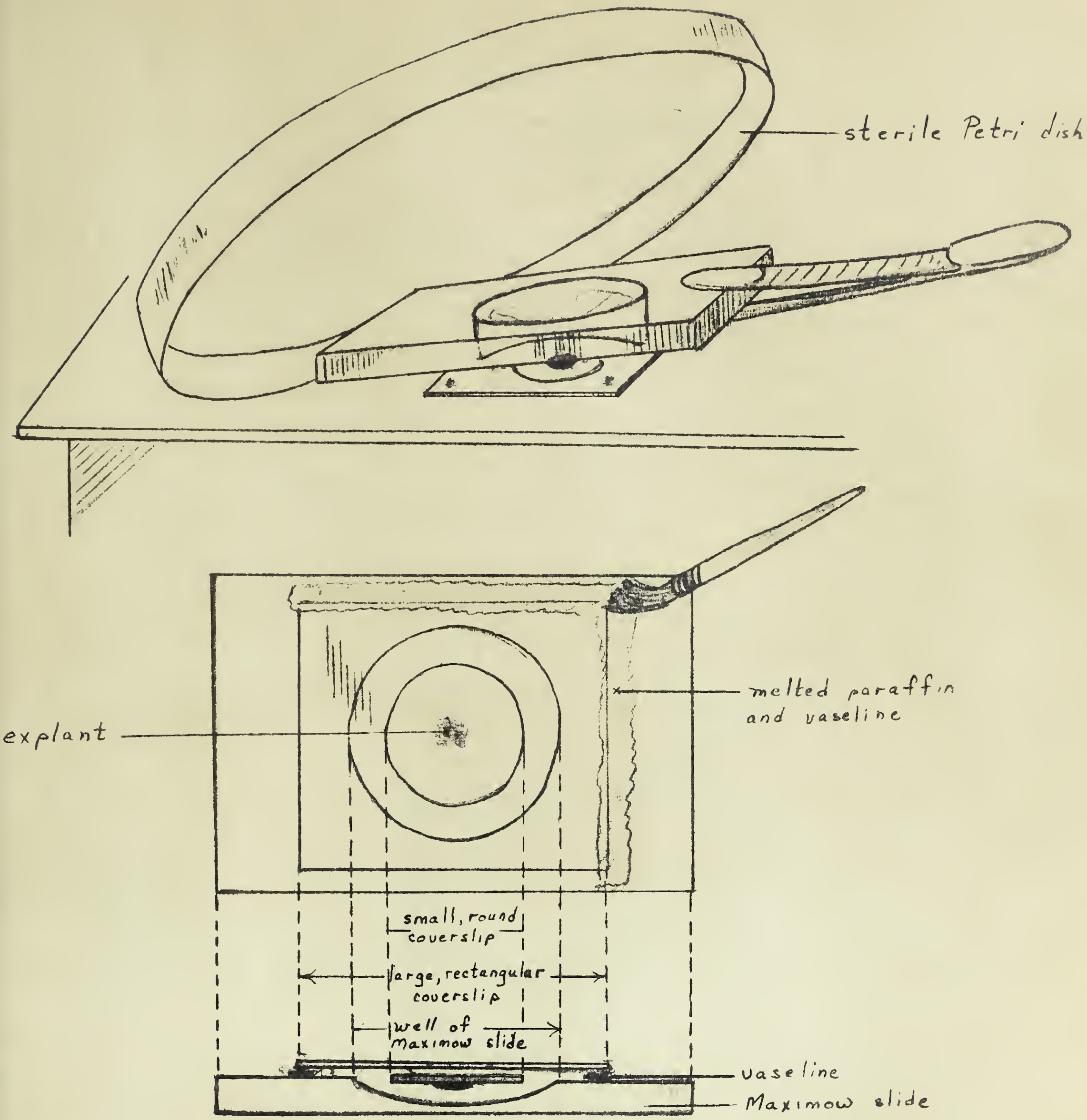
b. cultures for use under the phase microscope.

a. Standard Maximow cultures: (see Fig. 1)

Using aseptic technique and working under cover of a sterile Petri dish, a small, round coverslip was affixed to a larger rectangular one by an intervening layer of saline. A drop of plasma (prepared from dehydrated Difco plasma) was then placed on the small coverslip and spread out to form a thin plasma bed, on which were set several drops of previously prepared 50% 10 day chick embryo extract (50% Tyrode), and the liver explant. Vaseline was dotted on the corners of the large coverslip and the Maximow slide lowered over it, the well covering the culture. After several minutes, when the plasma had set, this unit was reversed through 180 degrees so that the culture now hung down into the well of the Maximow slide, and the large coverslip was sealed down with a mixture of melted paraffin and vaseline. This method was used for observing growth over an extended period of days.

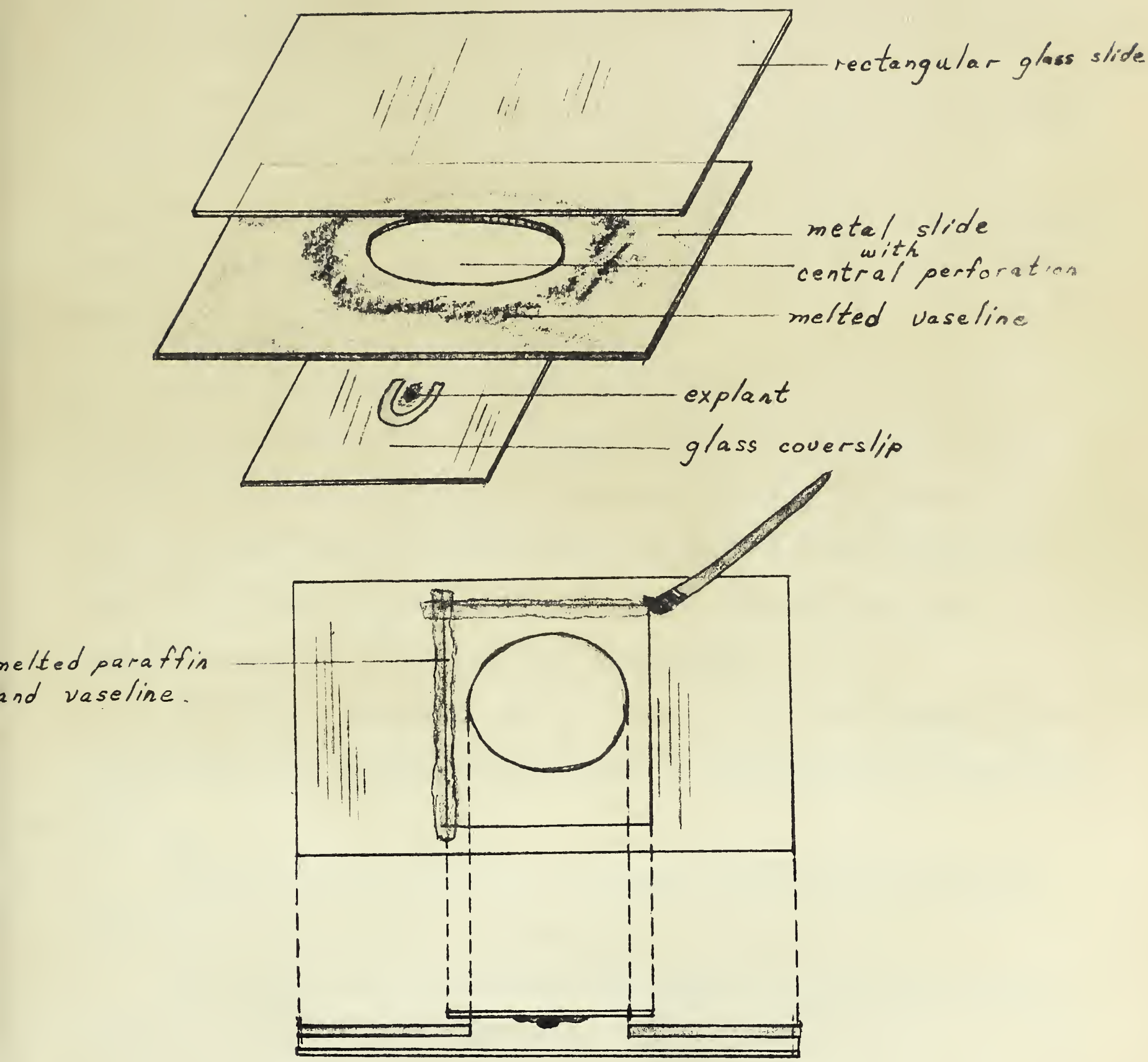
b. Phase cultures: (See Fig. 2)

The concave well of the Maximow slide acted as an additional lens, interfering with the optical system of the phase microscope. A new slide unit was devised, therefore, for use with phase microscopy. This consisted of a rectangular glass slide, a metal slide of identical size but with central perforation or well, and a smaller rectangular glass coverslip. The glass slide and perforated metal slide were sealed together by a ring of melted vaseline. This unit was then lowered over a rectangular coverslip carrying the explant, so that the well lay over the tissue.



MAXIMOW CULTURE

FIGURE 1.



PHASE CULTURE

FIGURE 2.

After allowing time for the plasma to set, the unit was reversed and the coverslip edges sealed with the paraffin-vaseline mixture. The shallowness of the chamber so created, presented a technical difficulty in reversing the unit, as a drop of fluid would form touching the two surfaces and quickly drain off to the side. To overcome this difficulty, in later cultures a horseshoe of alternate drops of plasma and 50% embryo extract was formed on the coverslip, mixed with a stirring rod, and allowed to clot in the incubator for five to ten minutes. A liver explant culture was set up within the horseshoe. This method effectively limited the loss of fluids.

Since the phase cultures were intended primarily for immediate observation of individual cells, a smear culture was attempted in place of the traditional tissue explant. The cut surface of embryonic liver was very slowly and gently drawn across the surface of the plasma bed. Observation showed groups of cells deposited in a manner that was admirably suited for individual cell observation.

All cultures were incubated at 37 deg. C. and were examined under a microscope which was housed in a 37 deg. C. thermostatically controlled unit.

Maximow cultures were fed at intervals of three to four days as indicated by the condition of the tissue.

For further microscopic study both types of cultures were washed, fixed, and stained. See Procedure 5.

A series of Maximow cultures of embryos ranging in age from 12 to 14 days was fixed and stained.

METHOD FOR STAINING WHOLE CULTURES FOR BEST NUCLEAR DETAIL

(Devised by Dr. W. Jacobson, Strangeways Laboratory
Cambridge, England.)

1. Wash 20 - 30 minutes in warm tyrode.
2. Fix cultures 5 minutes in methyl alcohol.
3. Stain 10 minutes in May-Grunwald's stain.
4. Stain 20 minutes in dilute Giemsa.
   (1:10 or 1:15 in distilled water).
5. Rapidly dehydrate in two changes of acetone
   and acetone-xylol (equal parts).
6. Clear in xylol and mount.

PROCEDURE 5.

IV. Tissue Smears:

Smears were taken of the freshly dissected embryonic livers. Either the whole liver, or the cut surface of a dissected portion, was smeared over the surface of a clean slide. This procedure was attempted with dry slides, slides newly dipped in methyl alcohol, and slides carrying a drop of normal saline. The liver was gently touched on the surface, producing a daub, and then in another area, very carefully and exceedingly slowly drawn over the surface of the slide, producing a smear. Some difficulty was encountered with the cells in the centre of the daub and smear. The slow evaporation of fluids at this spot seemed to increase the tonicity of the bathing tissue fluid, causing the cells to shrink. Smears on dry slides, if stood on end and dried immediately, gave good results. Saline smears were totally unsuccessful due to increasing tonicity with evaporation of fluid. The best results were obtained with the dry smears. After smearing the tissue on the alcohol-drained surface, the slides were immediately reimmersed in the methyl alcohol. Smears were stained in May-Grunwald-Giemsa stain modifying the time as follows: (May-Grunwald - 4 minutes: Giemsa - 16 minutes) at a pH of approximately 7.

V. Vital Stains:

A vital stain containing neutral red and pinacyanole was tried in several of the phase cultures in an attempt to bring out cell differences in the living material and thus facilitate microscopic study. The stain was prepared as outlined by Schwind (1950). Sterile stock solutions of neutral red (0.2 Gm. in 50 cc. absolute alcohol) and pinacyanole (0.05 Gm. in 50 cc. absolute alcohol) were prepared, and the diluted mixture made

up at the time of culturing. In culturing, the coverslip surface which would carry the smear was flooded with the diluted stain (30 drops stock neutral red to 9 drops stock pinacyanole, in 5 cc. absolute methyl alcohol). The plasma bed and liver smear were then prepared as usual on the dry, stained coverglass.

Results obtained with the vital stain were disappointing. Using the method outlined above, the explant became deeply colored with the neutral red, but individual peripheral cells and the migrating cells exhibited no visible staining. Further investigation of the literature revealed the statement that "the chief advantage of the supravital method over the smear technique therefore appears to lie in the fact that cells can be studied in the living condition, not from the viewpoint of identification on the basis of morphology, but because alterations in the physiological processes of those cells may be indicated, as judged by altered reactions to neutral red." (Hall, 1938) Since morphological identification was not enhanced, the method was abandoned.

OBSERVATIONS

1. Animals:

Out of thirty-two females killed, twenty-seven were pregnant. From these pregnancies, ninety-five embryos were obtained, and eighty-four of these were used in the preparation of sections, cultures, and smears.

Embryonic age was calculated on the basis of vaginal plug discovery, as described in the section on Materials and Methods, and crown-rump measurements were taken of most embryos. Embryos used for tissue explants were not measured because of the necessity for maintaining sterile technique in tissue culture preparation. Therefore, in many cases, the mean length for any embryonic age was not a true average of all the embryos in the litter. As multiple litters were used in the preparation of material for each age group, averages were determined on the total number of embryos. Table 1 shows the comparison between embryonic age and embryonic measurement, and it will be seen that the crown-rump length increased steadily with age. It was felt that this almost linear increase in size established a rough check on the accuracy of our vaginal plug timing method. It will be noted that our measurements differed from those of Gruneberg (1943), set out in column four of Table 1. Gruneberg did his measurements on embryos fixed in utero to avoid the "jack-in-the-box" effect of young, gelatinous embryos, whereas our measurements were done on unfixed embryos, dissected free of their membranes. It is possible that the discrepancy in the two sets of measurements was due:

a) to the "jack-in-the-box" effect mentioned by Gruneberg, and

MEASUREMENTS OF MOUSE EMBRYOS

Age (days)	Number of embryos	Mean Length (mm)	Mean Length (Gruneberg, 1943)
9	4	3.4	1.9
10	10	5.7	3.7
11	10	7.7	5.9
12	20	9.6	7.5
13	17	11.4	8.8
14	13	12.7	10.5
15	7	14.1	12.7

TABLE 1.

b) to some degree of shrinkage associated with fixation.

II. Fixed and Sectioned Material:

A. System of Identification and Nomenclature: From stained sections of 13 day embryonic liver representative cell types were carefully drawn and their individual characteristics described, to form a basis of recognition for these cells in the study of further sections. These representative cell types, when compared with cells described in standard histology textbooks, were seen to include cells of the erythropoietic series, plus megakaryocytes and liver epithelial cells. The nomenclature used by Undritz (1952) was adopted for most of the erythropoietic series.

Recognition criteria for the cells encountered in mouse embryonic liver were as follows:

1. Erythropoietic Series:

   a. Pronormoblast

      1) cytoplasm - lightly basophilic
         cobwebby
         good rim
         margins often indistinct

      2) nucleus - distinct membrane
         oval
         slightly eccentric
         chromatin - distinct, coarse, dots
            suggestion of threads
            fairly scanty
            loosely arranged

nucleoli - 1 or more
large
unbordered
distinct
marginal in nucleus

b. Basophilic normoblast

1) cytoplasm - medium to deep basophilia
cobwebby or homogeneous
fairly thin rim
distinct margin

2) nucleus - distinct membrane
round
tends to be eccentric
chromatin - becoming dense, compact and indistinct in structure.
nucleoli - present or absent

c. Polychromatic normoblast

1) cytoplasm - polychromatophilic
homogeneous
fair rim
distinct margins

2) nucleus - distinct membrane
tends to be round
eccentric
small
thin, surrounding halo
densely basophilic
chromatin - dense, coarse and indistinct; appears radially arranged.
no nucleoli

d. Oxyphilic normoblast

1) cytoplasm - uniformly oxyphilic
abundant
distinct, smooth margin

2) nucleus - round
small
tends to be eccentric
densely basophilic
only slight suggestion of structure
thin, light surrounding halo
chromatin - dense, coarse and indistinct
nucleoli - none.

e. Erythrocyte

1) cytoplasm - intensely oxyphilic
homogeneous
distinct margins

2) nucleus - not present.

2. Megakaryocyte

1) cytoplasm - distinctly purple
often rather mottled
fair rim
indistinct margins often

2) nucleus - 1-4 (or lobules)
distinct membrane
round, oval, or irregularly twisted
chromatin - coarse dots
- scanty
nucleoli - 1 or more
often irregular

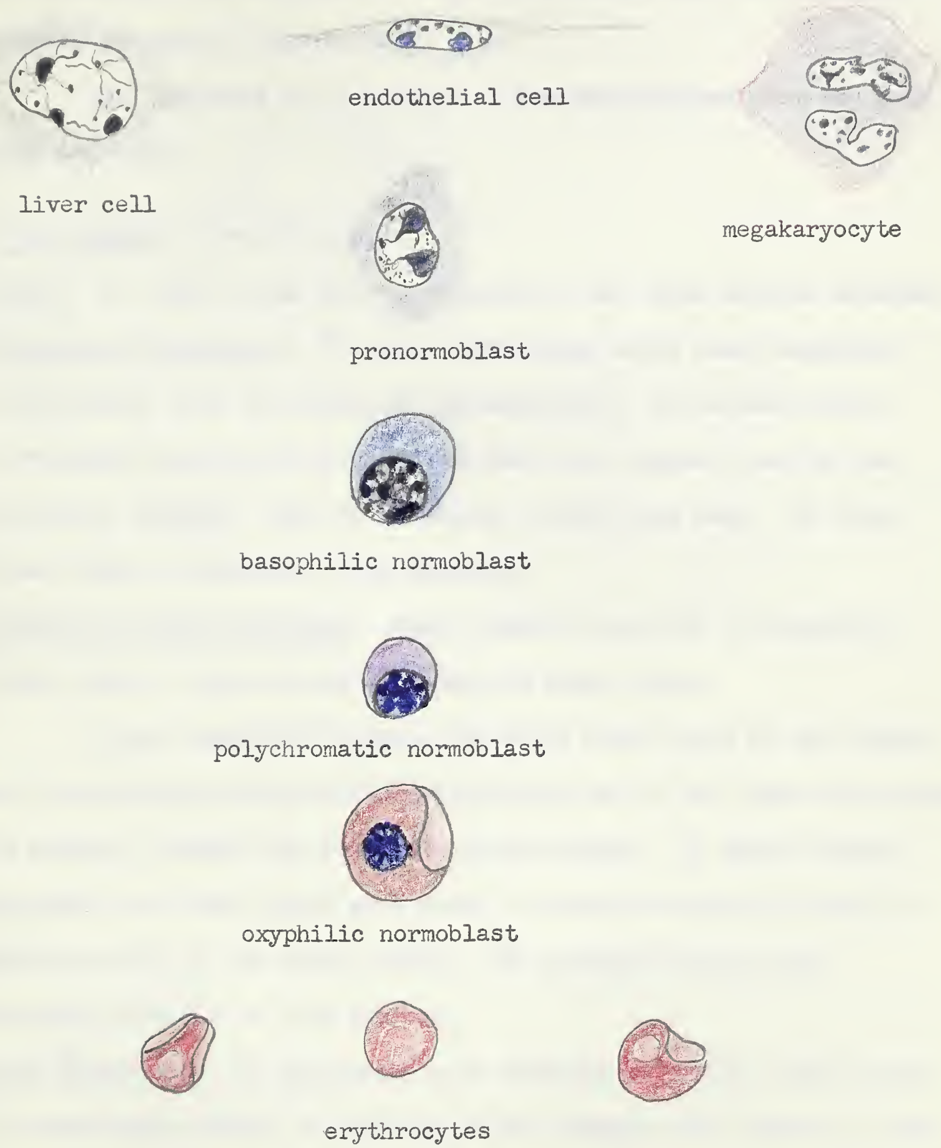
3. Liver Cell

1) cytoplasm - no basophilia
cobwebby & mottled
fair quantity
indistinct margins

2) nucleus - distinct margin
round, to oblong, indented
chromatin - fine dots and threads arranged peripherally; scanty and loose.
nucleoli - 1-3 large
distinct
central and peripheral

B. Observations of a series of mouse embryos: Stained sections were prepared of embryos ranging in age from nine to fifteen days. Microscopic examinations were made:

1. to determine a good proliferative age for red blood cells
2. to search for any data relevant to hemopoiesis.



CELLS ENCOUNTERED IN EMBRYONIC MOUSE LIVER

FIGURE 3.

Observations were systematically carried out (see specimen work sheets in Appendix), and differential blood cell counts done to facilitate greater accuracy in observation.

The following is an account of the observations made for each embryonic day.

9 DAY EMBRYO:- (see Fig. 4)

Liver: No liver tissue was encountered in the three embryos examined.

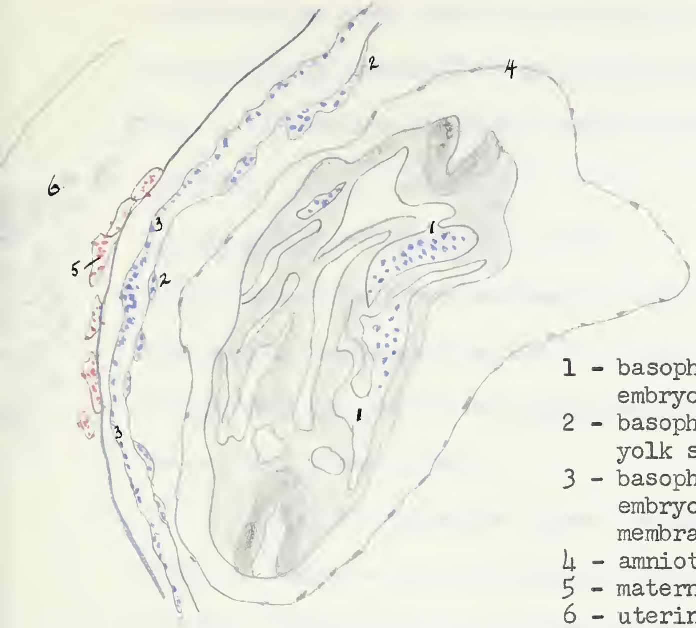
Embryonic Circulation: The only circulating cells were basophilic normoblasts, with an occasional pronormoblast. The actual number circulating was very small, both in the heart chambers and in the embryonic vessels. Only an occasional mitosis was seen. No white blood cells or platelets were observed.

Vessels of Fetal Membranes: These vessels were full of basophilic blood cells. Many mitoses were seen in these cells.

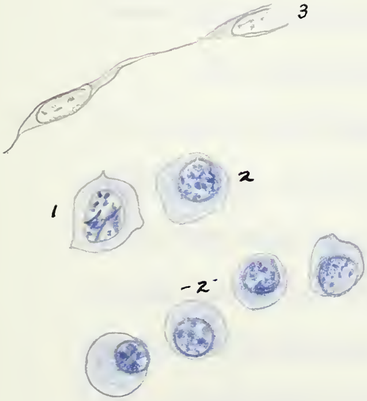
A good comparison between the adult blood cells of the mother and the embryonic blood cells was possible due to the close association of membrane vessels and placental blood sinuses. In these sinuses, the adult red blood cells were found to measure between 4.5 and 5.6 microns while in the fetal vessels, the embryonic blood cells measured from 8.4 to 11.2 microns.

Body Mesenchyme: It was possible to identify basophilic blood cells in mesenchymal spaces in the body of the embryo, even though no trace could be found of endothelium lining these spaces. Both single cells and small groups of cells were observed. The rounded basophilic cells stood out clearly among the fixed stellate mesenchymal cells.

Differential Counts: 10 counts of 100 cells each showed that basophilic



1 - basophilic blood cells in embryonic vessels
2 - basophilic blood cells in yolk sac vessels
3 - basophilic blood cells lining embryonic surface of Reichert's membrane
4 - amniotic membrane
5 - maternal erythrocytes
6 - uterine decidua



1 - pronormoblast
2 - basophilic normoblast
3 - endothelial lining of a vessel

9 DAY MOUSE EMBRYO

FIGURE 4.

normoblasts constituted 97 to 100% of the blood cells identified, the remaining small percentage being pronormoblasts. Mitoses averaged only around 3% of the cells counted.

Fig. 4 illustrates the typical blood picture of the 9 day mouse embryo.

10 DAY EMBRYO - (see Figs. 5 to 8)

Liver: Strands of liver epithelial cells and large, basophilic blood cells surrounded relatively large, endothelial-lined spaces filled with basophilic and polychromatic normoblasts, making up a loose, open liver tissue.

While the sinusoidal spaces contained a wide developmental range of cells -- pronormoblasts to polychromatic normoblasts -- the cells studded throughout the liver epithelial strands were only of the earliest type, i.e. pronormoblasts.

No megakaryocytes, white blood cells, or platelets were seen.

Mitoses were fairly frequent among free blood cells, and were occasionally encountered in the endothelial cells lining the sinusoids.

(One large embryo presented an atypical picture, corresponding to a much later stage of development.)

Circulation: Both in the vessels of the fetal membranes and of the body, the cells seen were, in the main, basophilic normoblasts and polychromatic normoblasts, with a few pronormoblasts and an occasional oxyphilic normoblast. Occasional mitoses were seen in basophilic and polychromatic normoblasts.

In the vessels examined, there was occasional evidence of endothelial proliferative activity -- i.e. occasional endothelial

mitoses; bulging endothelial cells; large, basophilic cells lying against the endothelial lining with cytoplasm flattened and apparently continuous with the endothelial lining.

In one embryo, the endothelial lining of the left atrium of the heart showed marked activity, many of the cells being swollen and bulging into the lumen. An occasional mitosis was seen in these cells, but there was no conclusive evidence that these cells were giving rise to new blood cells.

Body Mesenchyme: Spaces in the loose body mesenchyme contained both basophilic and polychromatic normoblasts.

Differential Counts: The embryonic circulation, both of the body and of the membranes, showed a preponderance of basophilic normoblasts (89 to 99%) with only a small percentage of pronormoblasts (1 to 8%) and of polychromatic normoblasts (0 to 6%).

The cells in the liver sinusoids were about equally represented -- i.e. approximately 33% each of pronormoblasts, basophilic and polychromatic normoblasts.

The extravascular blood cell population was predominantly pronormoblast (77 to 94%) with the basophilic normoblast making up the remaining small percentage.

Cell Measurements: Pronormoblasts averaged around 9 to 10 microns; basophilic normoblasts 7 to 8 microns; and polychromatic normoblasts 7 to 8 microns.

The occasional oxyphilic normoblasts encountered in the vessels of the embryo showed a wide divergence in cell diameter, smaller cells measuring 7 microns in diameter and larger cells

measuring up to 13 microns. No difference was detected in nuclear detail of these two extremes.

11 DAY EMBRYO - (see Figs. 9 and 10)

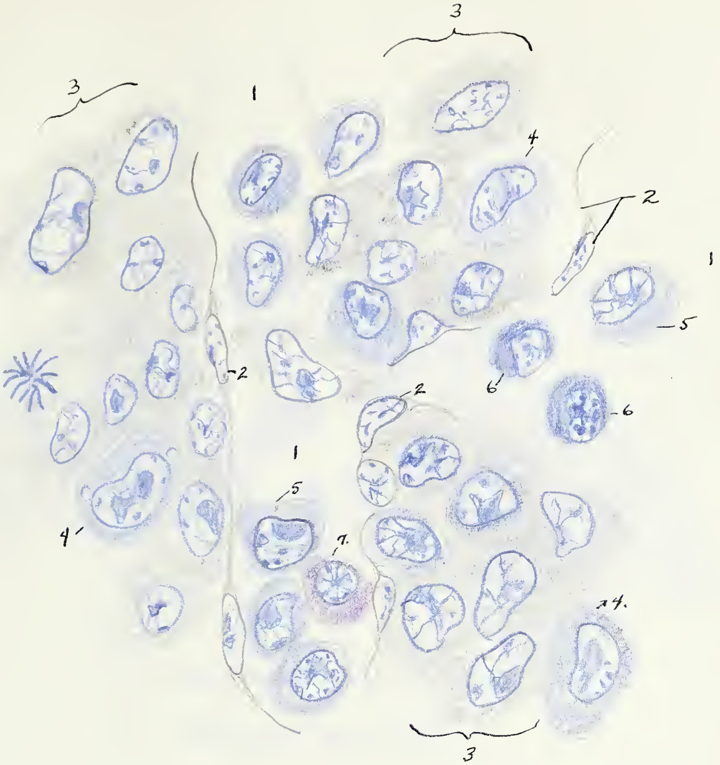
Liver: In the sections examined, liver structure varied from the loose, open tissue, characteristic of the 10 day embryo, to a more compact, densely cellular tissue, with endothelial-lined sinusoids obscured to a great extent by the extreme cellularity. In this more advanced liver, some large sinuses were seen and these contained oxyphilic normoblasts, predominantly.

On the whole, the cell population of the two areas, intrasinusoidal and extrasinusoidal, was distinctive. Sinusoidal counts showed a preponderance of basophilic normoblasts, with some pronormoblasts and oxyphilic normoblasts, and the liver strands were studded with pronormoblasts. Mitoses were observed in the basophilic cells in both areas.

No white cells or platelets were observed.

Oxyphilic normoblasts were observed occasionally alone or in small groups studded amongst the liver cells. Whether these were actually extrasinusoidal or whether the endothelial lining was just obscured in this section by the dense cellularity of the tissue was not clear.

An interesting feature of this liver was the grouping of the basophilic cells in the liver cords and strands. Whereas the pronormoblasts usually occurred alone or in pairs, it was quite common to see groups of basophilic cells which on inspection turned out to be basophilic normoblasts. Groups were usually even in number.



1 - liver sinusoid

2 - endothelium lining sinusoid

3 - liver epithelial cord

4 - extravascular pronormoblast

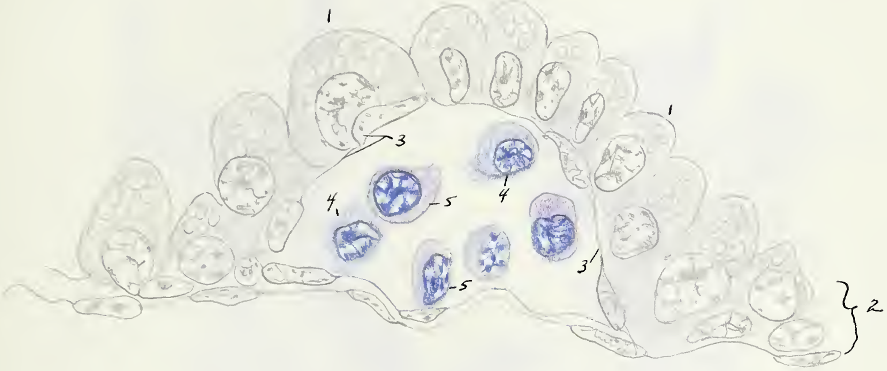
5 - intravascular pronormoblast

6 - basophilic normoblast

7 - polychromatic normoblast

10 DAY MOUSE EMBRYONIC LIVER

FIGURE 5.



1 - entodermal cells

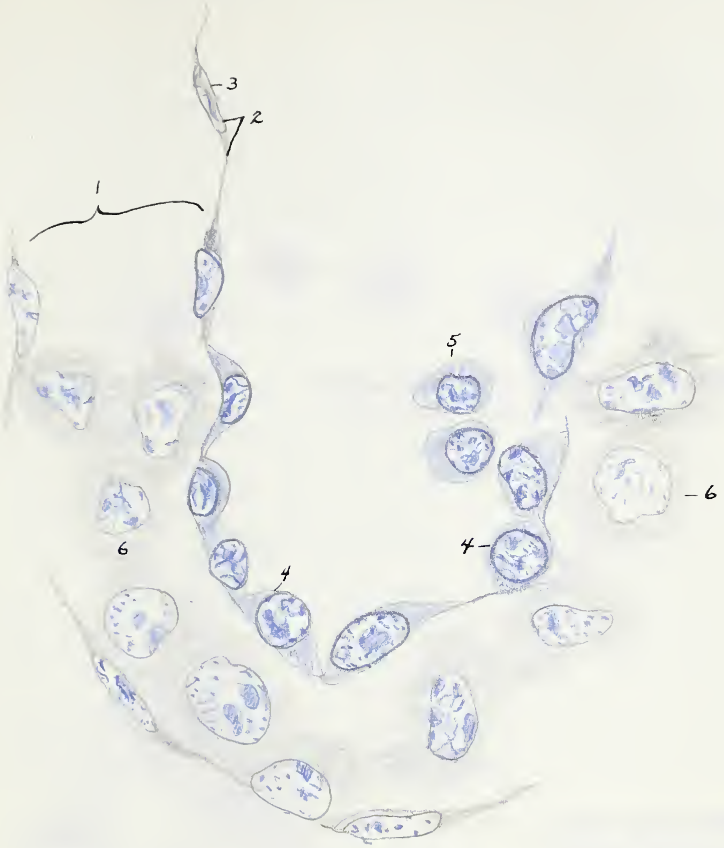
2 - embryonic yolk sac

3 - endothelial lining of yolk sac vessel

4 - basophilic normoblast

5 - polychromatic normoblast

YOLK SAC VESSEL - 10 DAY
FIGURE 6.



Left Atrium: 1 - wall of left atrium

2 - endothelial lining of atrium

3 - endothelial cell

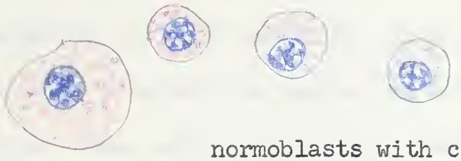
4 - bulging endothelial cell with basophilic cytoplasm

5 - basophilic normoblast free in lumen

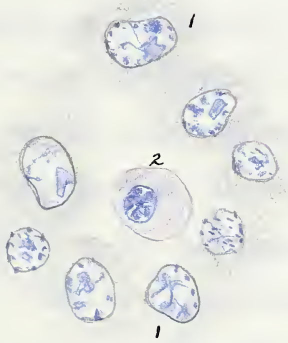
6 - mesenchymal cell of atrial wall (myocardial primordium)

ENDOTHELIAL ACTIVITY - 10 DAY

FIGURE 7.



normoblasts with cobwebby cytoplasm
(in embryonic vessel)



1 - loose mesenchymal tissue of 1st visceral arch

2 - polychromatic normoblast in unlined mesenchymal space

NORMOBLASTS - 10 DAY

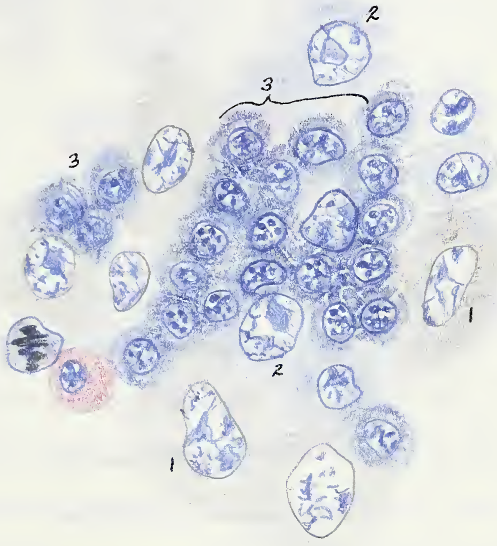
FIGURE 8.

A new feature noted in these liver sections was the presence of megakaryocytes. These occurred singly or in pairs and were often seen in close association with a sinusoid, although never seen within such a vessel. They were usually surrounded by a narrow, clear, surrounding zone. No evidence of platelets was observed. The megakaryocyte nucleus varied from a single, large, vesicular nucleus with prominent nucleolus, to a bilobed and multilobed condition. Some cells seemed to contain multiple nuclei. Occasional mitoses were observed.

Circulation: Basophilic normoblasts with a few pronormoblasts were typical of the embryos with the more immature liver structure, while in the more advanced embryo, the main circulating cell was an oxyphilic normoblast. Occasional mitoses were seen in these cells.

Differential Counts: The embryonic circulation showed a preponderance of basophilic normoblasts (89 to 98%) in the less mature embryos, and a preponderance of the same magnitude of oxyphilic normoblasts in the more mature embryos. A similar discrepancy was noted in the cell population of the sinusoids; the less mature containing approximately 70% basophilic normoblasts (the remainder being pronormoblasts), and the more mature liver containing up to 90% oxyphilic normoblasts (the remainder being basophilic and polychromatic normoblasts). In the extrasinusoidal areas, however, the preponderance of early cells (70% pronormoblasts to 30% basophilic normoblasts) was notable in both developmental stages.

Cell Measurements: The immature cells measured approximately the same as in earlier stages, while the oxyphilic normoblast measured



Extrasinusoidal Tissue:

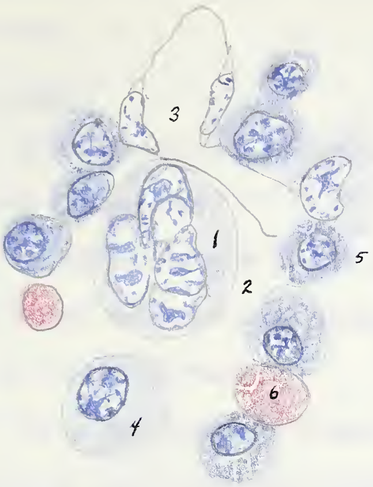
1 - liver epithelial cell

2 - pronormoblast

3 - clustered basophilic normoblasts

11 DAY MOUSE EMBRYONIC LIVER

FIGURE 9.



1 - polymorphonuclear megakaryocyte

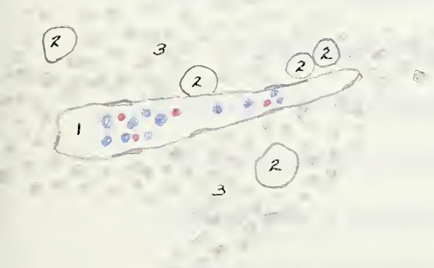
2 - clear surrounding zone

3 - nearby sinusoid

4 - pronormoblast

5 - basophilic normoblast

6 - erythrocyte



1 - sinusoid in liver

2 - megakaryocyte

3 - basophilic normoblasts

MEGAKARYOCYTES - 11 DAY

FIGURE 10.

approximately 7 to 8 microns and the megakaryocyte approximately 14 microns.

12 DAY EMBRYO

Liver: This organ showed some division into lobes, and was still intensely hemopoietic.

The sinusoids were packed with cells representing the whole developmental range of erythropoiesis, with oxyphilic normoblasts being the most numerous.

Due to the congested condition of the sinusoids, it was difficult to study individual cells. However, it was observed that the shape of the erythrocyte varied from spherical to concavo-convex, to biconcave. No suggestion of cytoplasmic structure was observed. A few loose nuclei (about 2 per 100 cells counted) were observed, and there were occasional suggestions of nuclear extrusion, with a loose nucleus lying in close proximity to an erythrocyte which still showed a depression formerly occupied by the nucleus. This depression was separate from the larger depressions giving the cell its concavo-convex or biconcave form.

Because of the dense cellularity of the tissue, it was difficult to delineate extrasinusoidal from intrasinusoidal areas. For this reason, observations and counts were strictly limited to those areas where a portion of endothelial lining could be detected clearly demarcating the two areas. In practice, this meant that counts were done on the larger sinusoids and on the extravascular tissue surrounding them. Large areas of the sections were thus ignored because of the impossibility of deciding in which category they belonged.

In the extrasinusoidal tissue, only the early stages of erythopoiesis were represented. Basophilic normoblasts predominated over pronormoblasts. (The 12 day sections were decidedly inferior to those of other days in terms of cellular detail, and especially in nuclear detail, and it may have been that the nucleoli and finer structure of the pronormoblast nucleus were obscured in the staining). Megakaryocytes could be seen spotted throughout the tissue (about 4 to 7 per 100 cells counted), but no platelets or white blood cells were observed.

Circulation: The cells circulating in the body of the embryo were polychromatic and oxyphilic normoblasts, the latter predominating. No evidence was observed here, or in the liver sinusoidal lining, of any endothelial activity.

Differential Counts: In the liver sinusoids, oxyphilic normoblasts were most numerous (73 to 90%), while the erythrocyte (0 to 15%) and the basophilic normoblast (4 to 11%) accounted for most of the remaining cells. Small numbers of pronormoblasts (0 to 4%) and polychromatic normoblasts (0 to 6%) were present. No mitoses and only occasional loose nuclei were observed.

In the extrasinusoidal tissue of the liver, basophilic normoblasts (84 to 86%) predominated over pronormoblasts (7 to 12%).

13 DAY EMBRYO: (see Figs. 11 to 15)

Liver: The liver was now large and could be easily dissected from the body of the embryo. The sinusoidal spaces were quite apparent, but the liver cords were not clearly demarcated. This organ was still intensely hemopoietic.

1. Intrasinusoidal:
    - a. stages present:- All stages in the erythropoietic series were present, with the oxyphilic normoblasts in slight preponderance.
    - b. shape of adult red:- These were very irregular when closely packed in the sinusoids; isolated cells were spherical and concavo-convex.
    - c. cytoplasmic structure:- Occasional oxyphilic cells had a uniformly granular cytoplasm.
    - d. loss of nucleus:- Many brightly oxyphilic cells had pycnotic nuclei apparently falling out of the cells; these did not suggest active extrusion of the nucleus in most cases. Many loose nuclei were seen, but no evidence for phagocytosis of these nuclei was observed. In some cases, fragments of varying size occupied the space obviously formerly occupied by the nucleus; in some cases, these fragments were encountered in the cytoplasm. Some light, faded nuclei with indistinct borders were also observed.
    - e. mitoses:- These were most frequently observed in polychromatic normoblasts. Often in cells showing mitotic configurations, the cytoplasm was not clear and it was difficult, therefore, to designate the cell stage accurately.
    - f. endothelial activity:- No conclusive evidence was observed for the origin of red blood cells from endothelium. However, a typical endothelium was observed, i.e. swollen endothelial cells, endothelial cells with basophilic cytoplasm, and basophilic cells with bases apparently continuous with the endothelial lining.

2. Extrasinusoidal:

a. stages present:- Only early cells of the erythropoietic series were present, the basophilic normoblasts predominating over the pronormoblasts. Some polychromatic normoblasts were also observed.

No white blood cells were observed.

Megakaryocytes were encountered fairly frequently (usually about 1 to 5 per 100 cells counted), and as in earlier ages, they were most often observed in the immediate vicinity of a sinusoid. Both polymorphonuclear and multinuclear forms were observed. In the apparently multinuclear forms it was not possible to observe any interconnection between the nuclei even with careful focusing. The cytoplasm appeared purplish with our stain, but no granules were observed.

Circulation: In both fetal membrane vessels and body vessels, the main circulating cell was the oxyphilic normoblast, the erythrocyte being the next most numerous cell type. In the fetal membrane vessels, occasional evidence of endothelial activity was seen in mitoses (with the axis parallel to the sinusoidal membrane), and swollen, basophilic endothelial cells.

Observed in the vessels of the embryonic membranes were occasional oxyphilic normoblasts with finer, more loosely structured nuclei (in contrast to the densely pycnotic, unstructured nucleus of the usual oxyphilic cell).

Loose nuclei (1 to 8 per 100 cells counted) were encountered in the vessels of both areas.

Differential Counts: Intrasinusoidal liver counts showed a complete developmental range for the erythropoietic series. Oxyphilic normoblasts were most numerous (32 to 51%) with polychromatic normoblasts (10 to 29%) and basophilic normoblasts (18 to 30%) accounting for most of the remaining cells. Erythrocytes (8 to 17%) and pronormoblasts (1 to 7 %) were relatively scarce. Mitoses were encountered infrequently (1 to 3 per 100 cells counted) and loose nuclei were scattered throughout (about 1 to 10 per 100 cells counted).

Extrasinusoidal counts repeated the picture of earlier days with basophilic normoblasts accounting for the majority of cells (56 to 81%) and pronormoblasts (10 to 26%) and polychromatic normoblasts (3 to 19%) also being represented. Mitoses were slightly more frequent (2 to 9 cells per 100 cells counted) than in the sinusoids.

Circulation counts showed oxyphilic normoblasts to be most numerous (50 to 89%) with erythrocytes (5 to 47%) accounting for most of the remaining cells. Polychromatic normoblasts (0 to 6%) appeared occasionally.

Cell measurements: Multiple cell measurements were taken of the red blood cells both in the liver and in the circulation (in body and in fetal membranes). For the cells that were found in both situations (the more mature members of the erythropoietic series), very little discrepancy was found in cell size. Average cell measurements were: pronormoblasts - 9 by 8 microns; basophilic normoblasts - 8 by 7 microns; polychromatic normoblasts - 5 microns; oxyphilic normoblasts -

8 by 7 microns; erythrocytes- 6 by 6 microns. Many polychromatic erythrocytes were observed in the vessels of the fetal membranes and these averaged 4 by 4 microns, and were, therefore, somewhat smaller than the oxyphilic erythrocytes.

14 DAY EMBRYO:- (see Fig. 16)

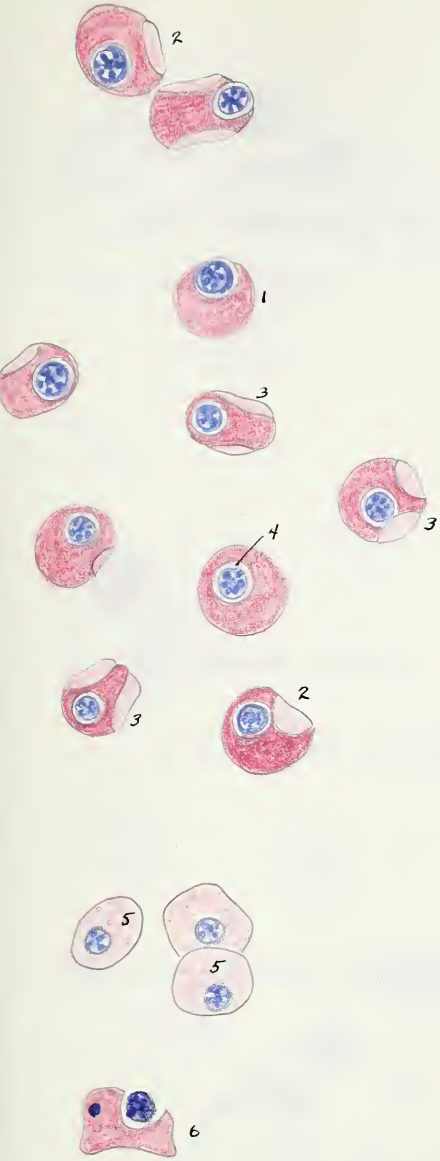
Liver: Division into lobes was observed, plus some division within lobes by long strands of connective tissue. There was an extensive sinusoidal network, but some sinusoids were smaller and more circular than the larger, more irregular sinusoids of younger embryos. The liver was still intensely hemopoietic, all the sinusoids being packed with oxyphilic cells.

1. Intrasinusoidal: All erythropoietic stages were represented, but erythrocytes were most numerous. Mitoses were not observed, and very few loose nuclei were seen (0 to 6 per 100 cells counted). The form of the erythrocyte and the mode of loss of the nucleus conformed to the picture seen in the 13 day embryo. No evidence was seen of endothelial activity.

   The polychromatic normoblasts were noted to have distinctly eccentric nuclei in these sections, and the cytoplasm usually showed very irregular margins, as though the cell were mobile.

2. Extrasinusoidal: As in earlier stages, only immature members of the erythropoietic series were observed. The majority of these were basophilic normoblasts.

   Megakaryocytes were spotted throughout the liver (1 to 3 per 100 cells counted) and usually lay in close proximity to a sinusoid, and often were surrounded by a clear zone. A new



1 - spherical

2 - cup-shaped, with single indentation.

3 - double indentation

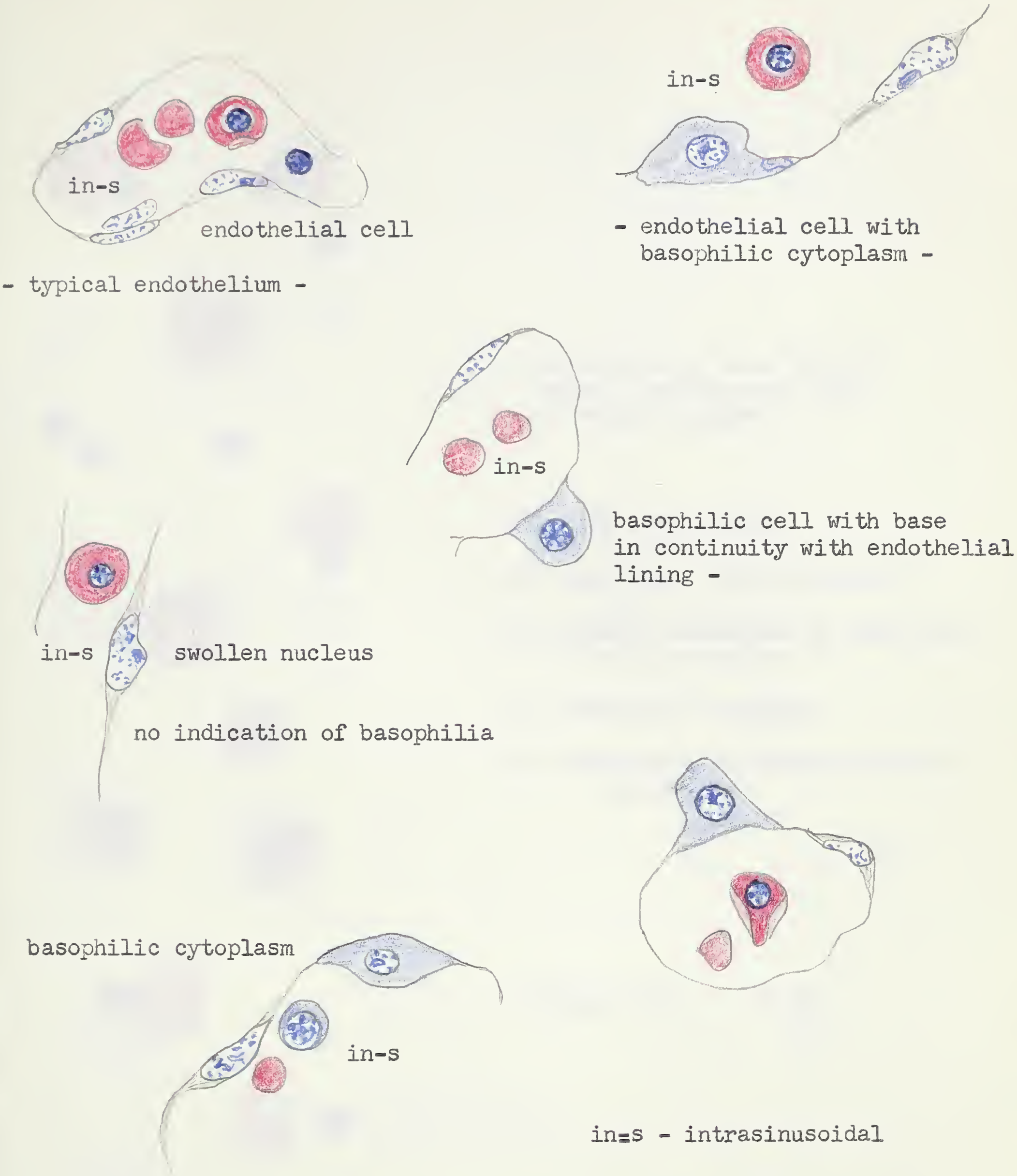
4 - clear halo surrounding pycnotic nucleus

5 - cobwebby appearance of cytoplasm

6 - oxyphilic normoblast with basophilic granule

OXYPHILIC NORMOBLAST - 13 DAY

FIGURE 11.



- typical endothelium -

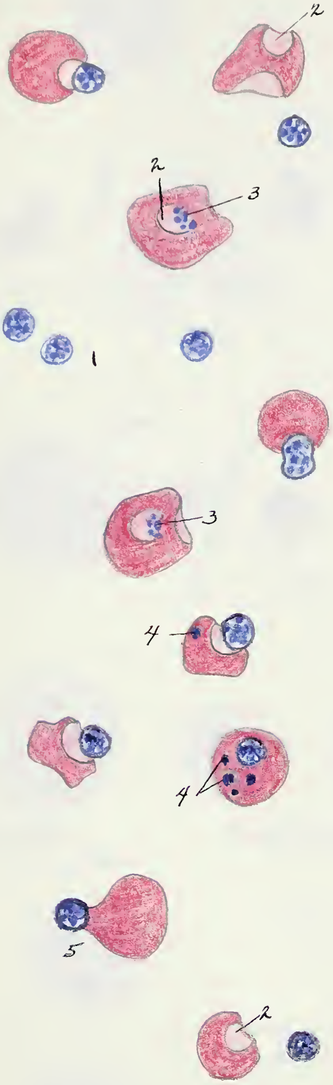
- endothelial cell with basophilic cytoplasm -

basophilic cell with base in continuity with endothelial lining -

in=s - intrasinusoidal

ENDOTHELIAL ACTIVITY - 13 DAY

FIGURE 12.



- appearance is more that of nuclei "falling out" than of being extruded -

1 - many bare nuclei

2 - depression left by nucleus

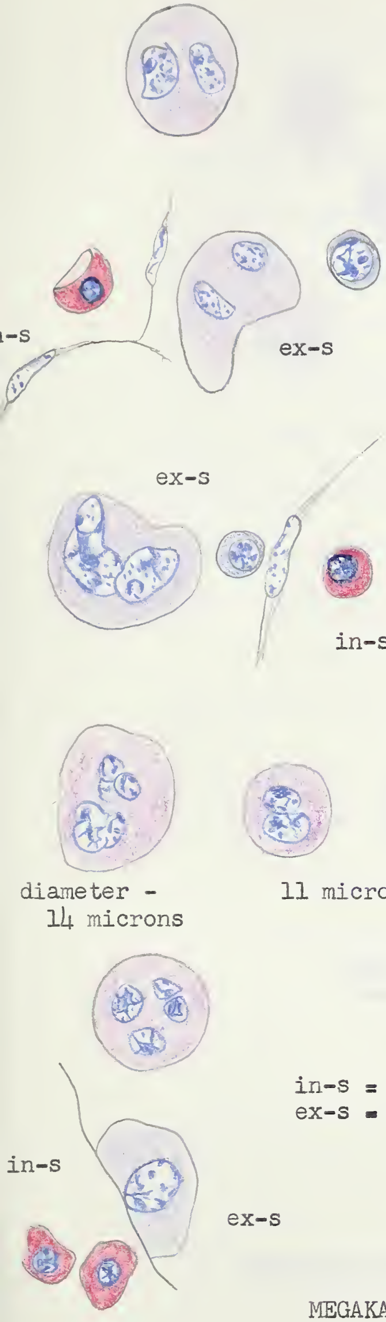
3 - nuclear particles in depression left by nucleus

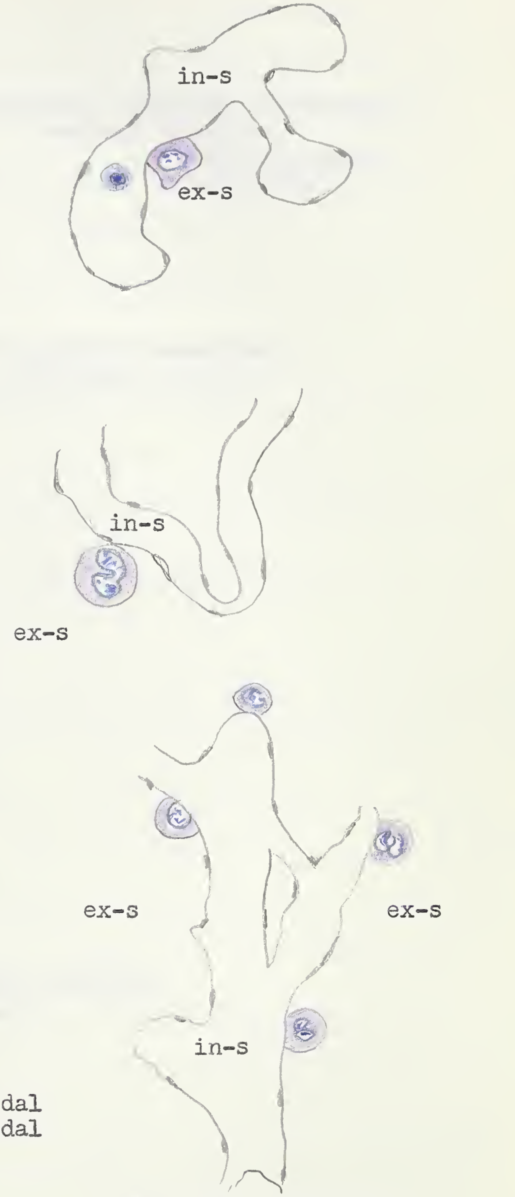
4 - basophilic granules

5 - appearance suggesting nuclear extrusion

NUCLEAR LOSS - 13 DAY

FIGURE 13.



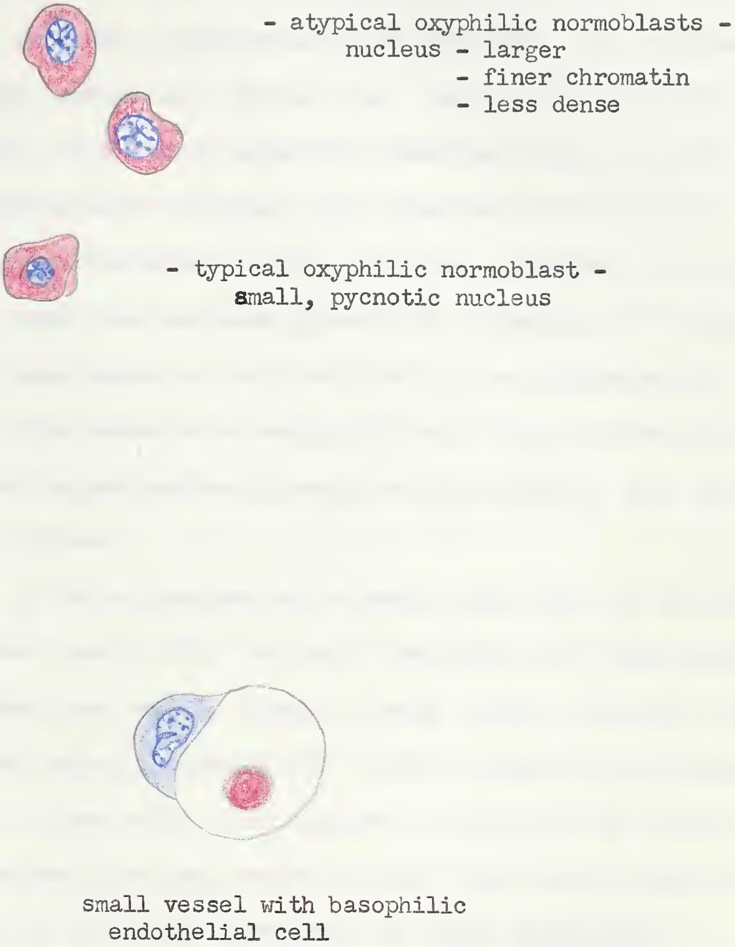


in-s = intrasinusoidal
ex-s = extrasinusoidal

LOW POWER APPEARANCE of Megakaryocytes associated with liver sinusoids.

MEGAKARYOCYTES - 13 DAY

FIGURE 14.


- atypical oxyphilic normoblasts -
nucleus - larger
- finer chromatin
- less dense
- typical oxyphilic normoblast -
small, pycnotic nucleus
small vessel with basophilic
endothelial cell

EMBRYONIC MEMBRANE VESSELS - 13 DAY

FIGURE 15.

phenomenon was observed in the suggestion of bubbling of the megakaryocyte cytoplasm -- this could be observed both at the cell margins, as cytoplasmic protrusions, and within the cytoplasm, as darker, globular areas of cytoplasm. The cytoplasm in these bubbles was a darker blue. Small (2 by 2 microns), globular, blue bodies, apparently identical with the bubbles of the megakaryocyte cytoplasm, were observed in the immediate vicinity of the megakaryocytes, and were considered to be platelets.

Some pronormoblasts appeared to be budding off cytoplasm in the same manner as that described for the megakaryocyte. These round, blue masses were usually the same size as those associated with the megakaryocytes although one such globular mass measured 5 by 5 microns.

In the extrasinusoidal tissue, a new cell type was encountered. This was a small (5 by 5 microns) basophilic cell with sparse cytoplasm, and coarse, clumped, cloudy nuclear chromatin, which occurred rather frequently (30 to 50% of counts) in the extravascular tissue. These cells often occurred in groups and we called them lymphocytes since they seemed distinct from the red blood cell series and fitted the description of small lymphocytes.

Circulation: Erythrocytes were the chief circulating cells, with a smaller percentage of oxyphilic and polychromatic normoblasts also present. Some of the erythrocytes showed a polychromatic cytoplasm.

Differential Counts: Intrasinusoidal counts showed the erythrocyte (61 to 73%) to be the most numerous cell type, while the oxyphilic

normoblast (13 to 34%) was next. The less mature cells were also represented; polychromatic normoblasts (0 to 8%), basophilic normoblasts (1 to 12%), and pronormoblasts (0 to 3%). Loose nuclei were occasionally encountered (0 to 6 per 100 cells counted).

Extrasinusoidal counts showed the familiar preponderance of basophilic normoblasts (25 to 60%) over pronormoblasts (0 to 12%) and polychromatic normoblasts (0 to 4%). Only occasional mitoses were observed (1 per 100 cells counted). The rest of the cell population was made up of the new, small, basophilic cells we have termed lymphocytes, these representing anywhere from 34% to 67% of the cells counted in this area.

Circulation counts showed the erythrocyte (64 to 79%) to be the main circulating cell, while two younger cells, the oxyphilic normoblast (12 to 26%), and the polychromatic normoblast (3 to 14%) were also present.

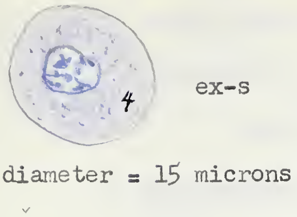
Cell Measurements: Individual cell measurements had not changed from the 13 day embryo.

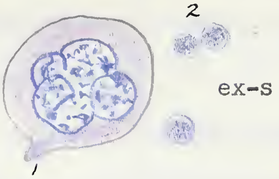
15 DAY EMBRYO:- (see Fig. 17)

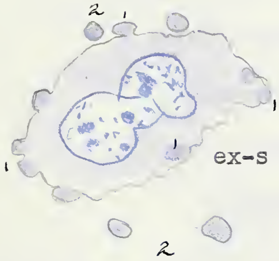
Liver: At this stage, there was some suggestion of adult structure, with cords of liver cells radiating out from a central vessel. The liver was still intensely hemopoietic.

1. Intrasinusoidal:- Erythrocytes and oxyphilic normoblasts were present in the greatest numbers, but polychromatic and basophilic normoblasts were also present.

   Particulate matter was observed in both erythrocytes





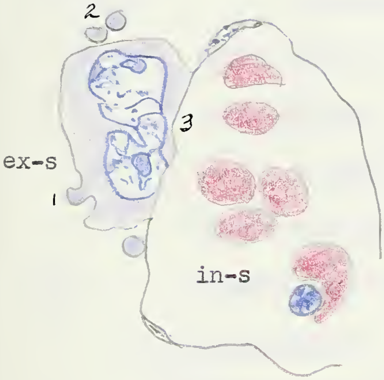


1 - bubbling megakaryocyte cytoplasm

2 - platelets

3 - common situation of megakaryocytes adjoining a sinusoid

4 - slight granularity of megakaryocyte cytoplasm



in-s = intrasinusoidal
ex-s = extrasinusoidal

MEGAKARYOCYTES AND PLATELETS - 14 DAY

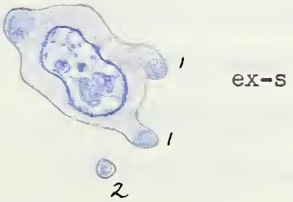


FIGURE 16.

and oxyphilic normoblasts. This varied from a single, small particle in an oxyphilic nucleated cell to multiple fragments in a non-nucleated cell.

Occasional nuclei appeared faded and ghost-like.

2. Extrasinusoidal: The familiar picture of immature cells only in this area was observed once more. The more numerous basophilic normoblasts were thickly studded throughout the intersinusoidal tissue and characteristically were arranged in groups of even numbers. The pronormoblasts on the other hand occurred singly or in pairs.

Megakaryocytes were present and there was some suggestion of eosinophilia and granularity of their cytoplasm.

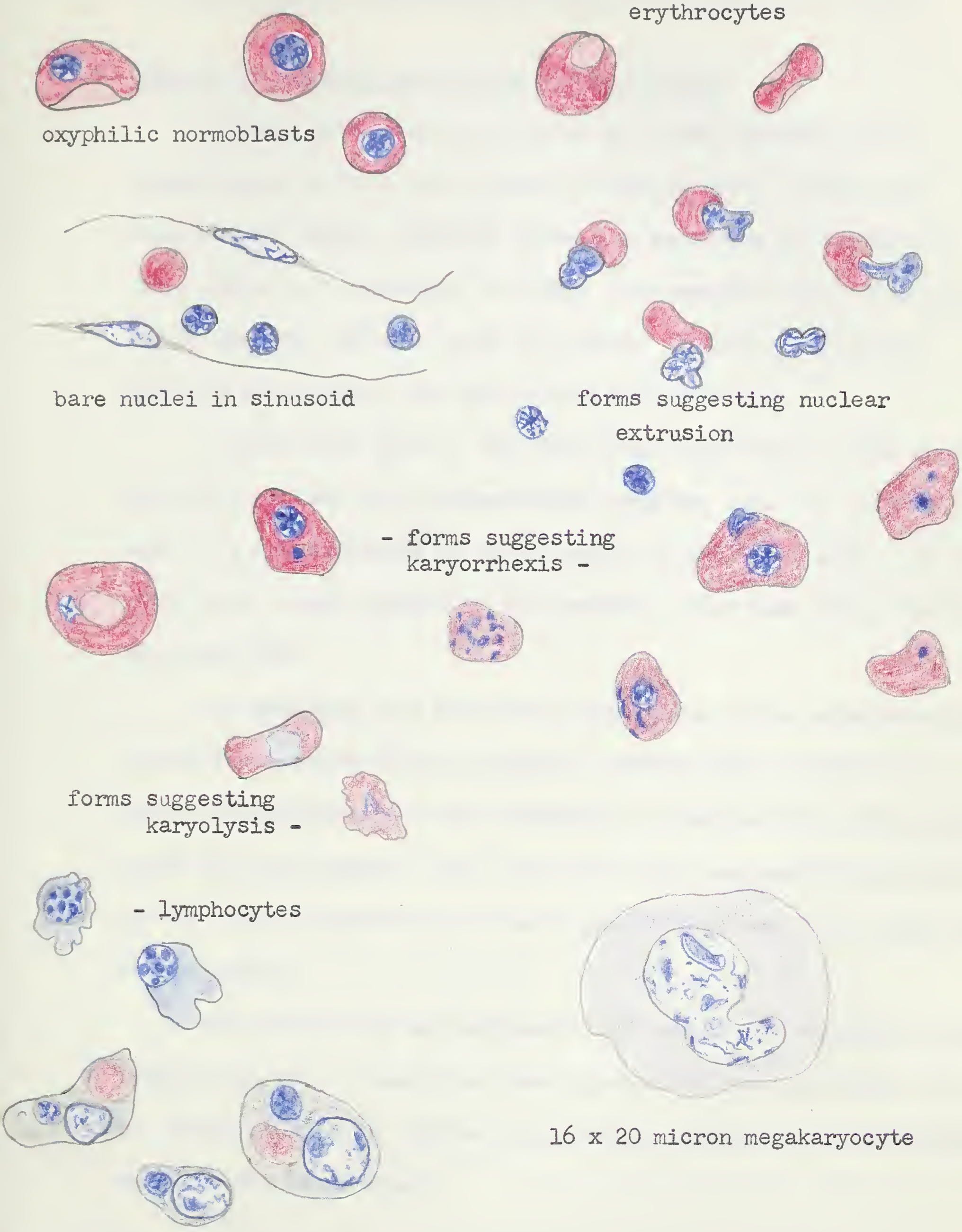
An occasional phagocytic cell, containing normoblastic nuclei with or without a hemoglobin-containing plastid, was also observed.

A small, basophilic cell with irregular, lightly basophilic cytoplasm and checkerboard nucleus, similar to our lymphocyte of the 14 day embryo, was noted.

Circulation: Only the liver was fixed for this stage, so observations on body or fetal membrane vessels were impossible.

Differential Counts: In the sinusoids there was an approximately equal representation of erythrocytes (38 to 49%) and oxyphilic normoblasts (35 to 47%), while polychromatic normoblasts (2 to 4%) and basophilic normoblasts (0 to 9%) made up the rest of the cell population.

In the extrasinusoidal tissue, basophilic normoblasts


erythrocytes
oxyphilic normoblasts
bare nuclei in sinusoid
forms suggesting nuclear extrusion
- forms suggesting karyorrhexis -
forms suggesting karyolysis -
- lymphocytes
16 x 20 micron megakaryocyte
- phagocytes -

15 DAY

FIGURE 17.

(76 to 89%) predominated over pronormoblasts (11 to 24%).

C. Overall development during the period studied:

During this seven day period the liver appeared, grew tremendously in bulk, and gradually changed from a loose open structure of large, irregular sinusoids separated by strands of liver cells to a structure of large, cell-packed sinusoids separated by densely cellular cords of tissue. Intense hemopoietic activity was evident throughout this entire period.

During this period, the blood cell population of the sinusoids gradually shifted from predominantly immature cells in the early ages to a preponderance of mature cells in the later ages. In the later ages, small numbers of the immature cells were still seen in the sinusoids.

In contrast, the blood cell population of the extrasinusoidal tissue remained relatively immature, showing only a shift to a greater preponderance of the basophilic normoblast over the pronormoblast in later stages. Also, over the seven day period, new cells, in the form of megakaryocytes and lymphocytes, made their appearance in this area.

The vessels of the embryonic body and of the embryonic vessels, likewise showed a transition from circulating immature blood cells of the erythropoietic series in the early ages to circulating mature cells in the later ages.

D. Determination of a good proliferative age to be used in tissue cultures:

It was obvious from a scrutiny of the observations made over

this developmental period that active proliferation was characteristic of any day. However, since we proposed to attempt to follow the maturation of a relatively immature cell, a day showing a preponderance of immature cells seemed to be indicated. While the 10 and 11 day livers showed the greatest percentage of basophilic normoblasts, it was almost impossible to grossly dissect out the liver in these ages. Some 11 day cultures were attempted, but the majority of cultures were prepared from embryos ranging in age from 12 to 14 days. At these ages, the liver was easily detectable, and it was felt that since the extrasinusoidal tissue remained relatively immature in its blood cell population, success in detecting and following an immature cell of the erythropoietic series was a reasonable expectation.

III. In Vitro Tissue Cultures:

Fifteen embryos of nine mice were used in preparing sixty-four cultures (40 Maximow cultures and 24 phase cultures) of liver of embryos ranging in age from 11 to 15 days. The majority of the cultures were prepared from 12 day embryonic liver.

A. Observations on Living Cultures: (See Fig. 18). These were observed intensively over a period extending up to 10 days on some cultures.

1. Identification of blood cells in the cultures: This proved to be a difficult problem. In the unstained living condition, the differential characteristics so clear in stained, sectioned material were absent, and new recognition criteria had to be determined.

Amongst the cells present in the outgrowth from the explant of liver tissue, round cells were noted to be distinctive from cells with marked development of cytoplasmic processes, and also from cells, roughly polygonal in shape which tended to form a sheet-like growth. We felt the round cells conformed more to the picture of blood cells as demonstrated in sections and smears.

Intensive observation of these round cells disclosed the following facts:

a. some cells were nucleated; others were non-nucleated.

b. the cytoplasm of some cells showed a peculiar yellowish color; this yellowish color varied in intensity in different cells; the yellowish color was most marked in the non-nucleated cells.

c. the cells varied in size.

On the basis of these observations we decided that at least some of the round cells were red blood cells, both nucleated and non-nucleated forms. Secondly, we decided that the varying intensity of the yellowish color of the cytoplasm could be due to the accumulation of hemoglobin in the cytoplasm, and could be compared to the increasing eosinophilia of the maturing red blood cell seen in stained, sectioned material. Therefore, on the basis of these assumptions we could designate the non-nucleated, yellowish round cell, an erythrocyte, and the nucleated yellowish round cell a normoblast - whether a polychromatophilic or an oxyphilic normoblast depending on the intensity of the yellowish

color of the cytoplasm. Also, round nucleated cells without any yellowish color could be basophilic normoblasts.

Having determined the above recognition criteria, intensive observation was carried out on all cultures of the normoblast in an attempt to follow its maturation.

2. Maturation of blood cells in the cultures: This proved to be a very disappointing part of this investigation. Although constant observation over a period of hours was carried out repeatedly on our series of cultures, very little was learned. What was observed can be summarized briefly.
    a. The yellowish color of selected cells was observed to increase.
    b. Erythrocytes were observed on occasion to display a continuing change of shape, numerous pseudopodia being extended and withdrawn.
    c. Although evidence suggestive of nuclear extrusion was observed, the actual phenomenon of nuclear extrusion was not seen. Suggestive evidence included: repeated bulging out and drawing in of the nucleus; nuclei apparently adhering to the edges of cells; small round bodies, suggestive of nuclei, occurring in the neighborhood of red blood cells.
3. Incidental Observations:
    a. Masses of red blood cells were observed in the periphery of the explant in cultures of embryos of the later ages. We felt that these cells had migrated out from the explant, since we had not observed cell division accounting for such a proliferation.

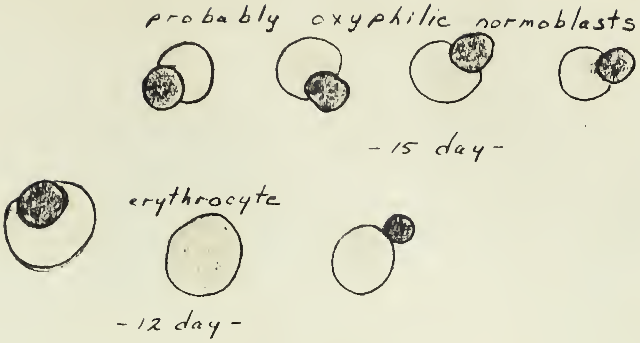
b. In the 13 and 14 day cultures especially, we observed that the blood cells occurred in clusters, usually 5 to 9 cells forming such a group. The cells within these groups differed in size.

c. The outgrowth from the liver explants was predominantly a sheet-like formation of cells, which we considered to be liver epithelial cells. Fibroblasts with cytoplasmic extensions, and endothelial cells, with extremely long cytoplasmic processes and a tendency for the processes of neighboring cells to be joined, were also observed.

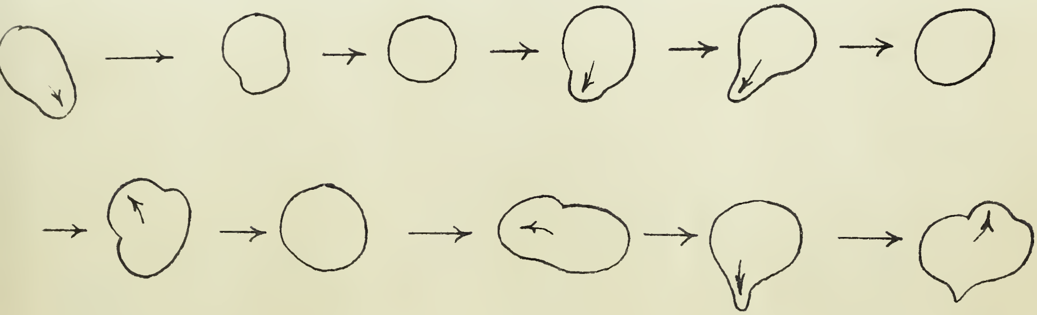
B. Observations on fixed and stained cultures: (See Fig. 19)

1. Outgrowth: This varied from no outgrowth to a band of outgrowth twice the diameter of the original explant. Individual cultures varied widely so that the outgrowth of a tissue cultured for eight days was not necessarily greater in extent than the outgrowth of a tissue cultured for four days.

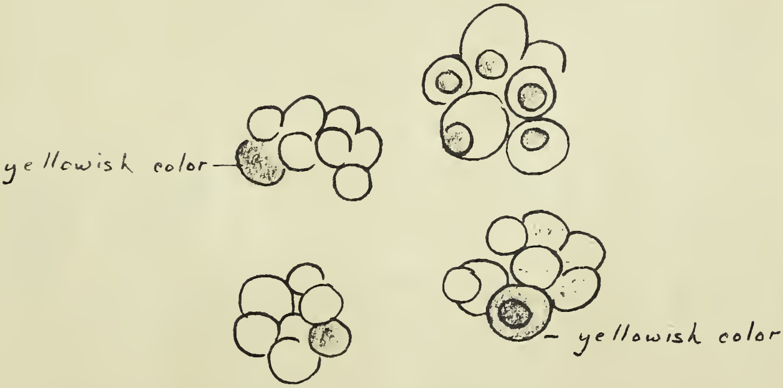
The outgrowth in the majority of cultures was in the form of an epithelial sheet of cells, which we felt to be liver cells. Also present in the outgrowth were cells with extensive cytoplasmic processes, which we felt to be fibroblasts, and cells with oval nuclei and cytoplasm pulled out into two long processes at opposite poles of the cell. These latter cells occasionally were aligned in such a manner as to suggest the formation of a tubular channel, and we felt they were endothelial cells. Large, round cells with granular debris and pycnotic nuclei within their cytoplasm were considered to be phagocytes.



Cells showing yellowish color in cytoplasm



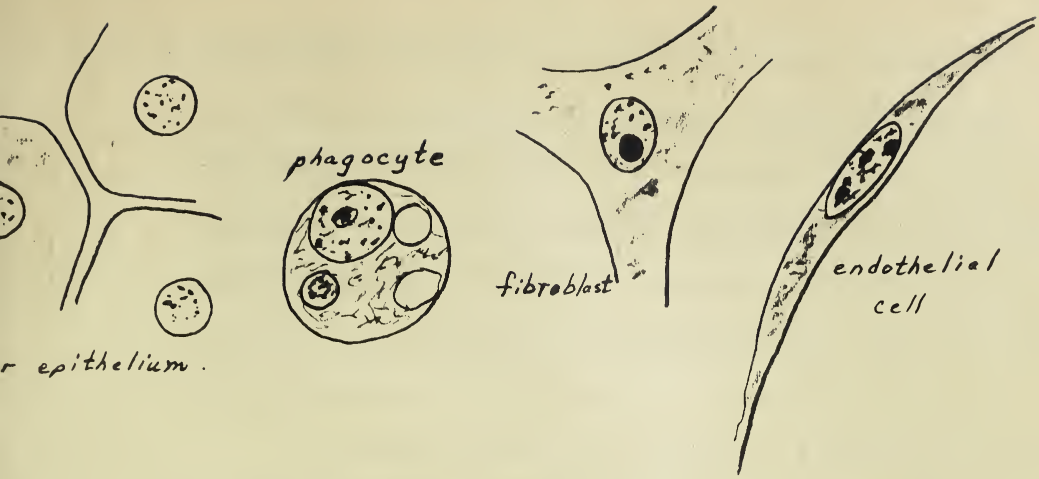
ange in shape of erythrocyte during 15 minutes observation
- 12 day -



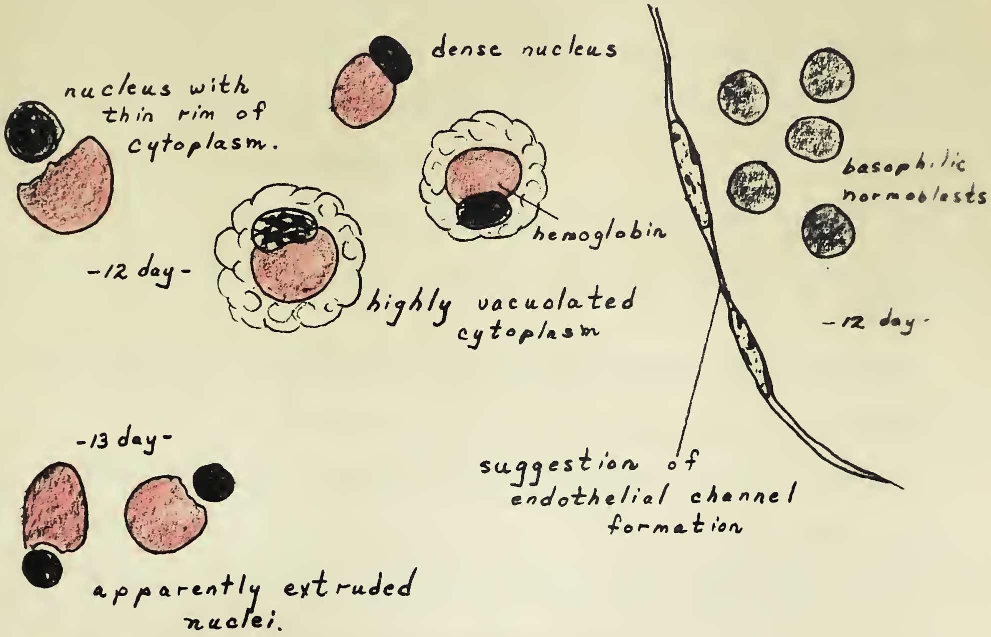
Typical cell clusters - 13 day -

OBSERVATIONS OF LIVING CULTURES

FIGURE 18.



Cell types seen in outgrowth zone
-12 day-


dense nucleus
nucleus with thin rim of cytoplasm.
-12 day-
hemoglobin
highly vacuolated cytoplasm
basophilic normoblasts
-12 day-
-13 day-
suggestion of endothelial channel formation
apparently extruded nuclei.

OBSERVATIONS OF FIXED AND STAINED CULTURES

FIGURE 19.

2. Blood cells: The number of blood cells present varied widely from culture to culture. On the whole, however, there were very few blood cells present, and these were usually in the immediate vicinity of the explant. The majority of blood cells seenwere basophilic normoblasts, with some polychromatophilic normoblasts and oxyphilic normoblasts present. Very few mitoses were observed, and these occurred in basophilic normoblasts.

In several cultures, the basophilic normoblasts occurred in clusters, while in other cultures they were scattered singly.

One stained culture showed oxyphilic normoblasts with pycnotic nuclei eccentrically placed, and hemoglobin compactly gathered in a globular mass adjoining the nucleus. Both the nucleus and the mass of hemoglobin were surrounded by a rim of highly vacuolated cytoplasm. This may have represented a form of degeneration.

Scattered examples were seen of pycnotic nuclei with a thin rim of ragged cytoplasm in close proximity to an erythrocyte.

C. Comparative results obtained with Maximow and phase cultures:

On the whole, phase cultures were more satisfactory for use with the phase microscope because of the elimination of the extra lens created by the well of the Maximow slide. For immediate observation of cells, the smear phase culture gave excellent results, but this type of culture deteriorated quickly after approximately 12 hours.

IV. Smears of Embryonic Liver:

A. Observations made on a series of days:

11 day embryo: A characteristic picture was observed. Cells were deposited on the slides in definite groups, and only cells of the erythropoietic series were observed.

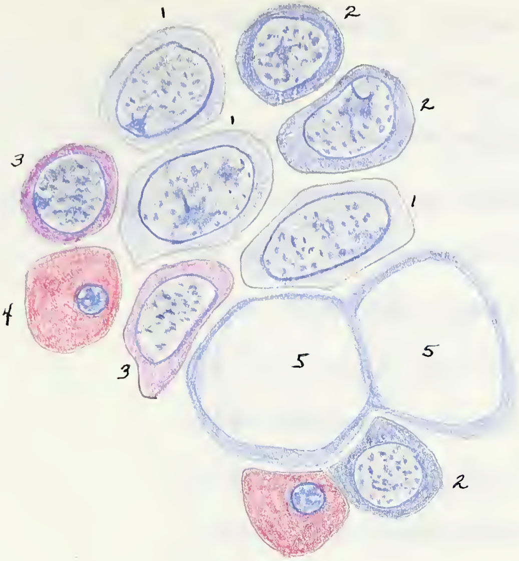
a. number of cells in a group: This varied from around 5 cells to around 50 cells. Small groups were more typical.

b. cell types in a group: Usually the entire developmental range of the erythropoietic series was represented; some groups did not contain oxyphilic stages.

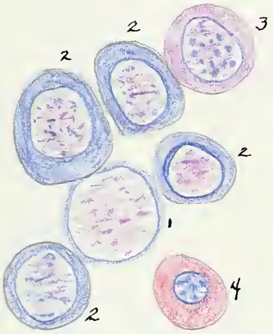
c. numbers of each type: Basophilic normoblasts were most numerous, and often these occurred in even numbers. One or two pronormoblasts were usually present. Oxyphilic and polychromatophilic normoblasts were few in number on the whole, although some small groups consisting mostly of oxyphilic normoblasts were observed.

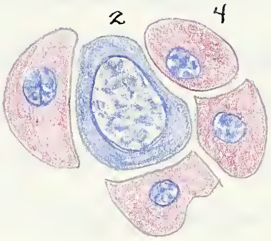
d. arrangement of cells within the groups: cells at the same stage tended to be adjoining; the more mature cells (e.g. oxyphilic normoblasts and erythrocytes) tended to occupy the peripheral part of the cell cluster.

e. additional observations: Mitoses were observed in all cell stages. Pronormoblasts did not take up the stain



- Typical cell clusters -





1 - pronormoblast

2 - basophilic normoblast

3 - polychromatic normoblast

4 - oxyphilic normoblast

5 - probably poorly stained pronormoblast

11 DAY LIVER SMEARS

FIGURE 20.

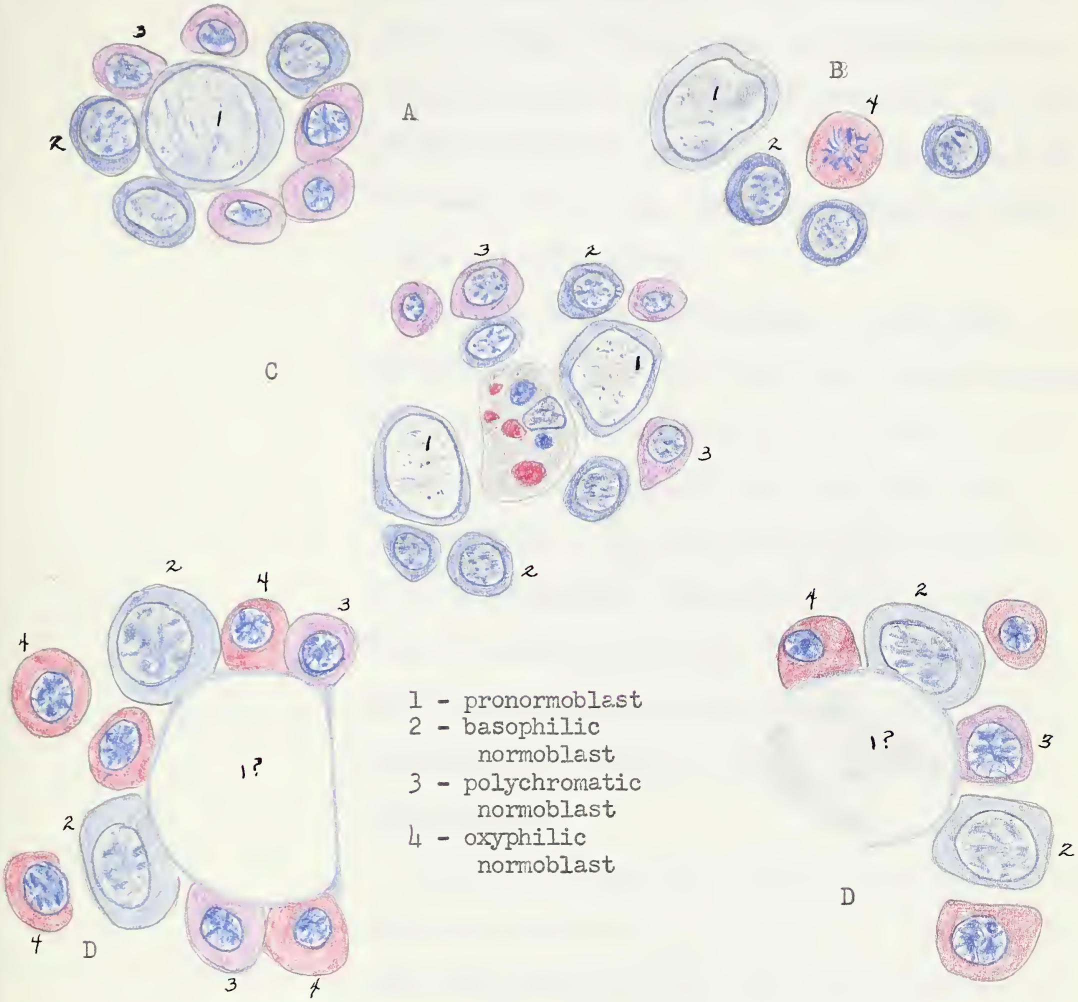
as well as the basophilic and oxyphilic cells. An empty, clearly outlined space was observed in many of the cell clusters. Often cells would be arranged around the periphery of this space.

12 day embryo: Cell clusters of the erythropoietic series were once again the prominent feature. As in the 11 day embryo, there was a great variance in the number of cells in a group, and the entire developmental range of the erythropoietic series was usually represented.

a. numbers of each type: In the 12 day embryo, the polychromatic normoblast was the predominant cell in most groups. Each developmental stage was usually represented by more than one cell, and like cells were grouped together in many cases.

b. arrangement of cell types within the groups: Cells seemed typically to be arranged around some central structure: this might be one or several pronormoblasts; a large phagocyte containing pycnotic nuclei and plastids; or a clearly outlined space (as observed in the 11 day embryo).

c. additional observations: Mitoses were observed in all cell types, but appeared to be most frequent in polychromatic normoblasts. Mitoses in oxyphilic normoblasts often appeared irregular or unorganized. Bare, pycnotic nuclei were observed in some cell clusters, associated or not with erythrocytes.



A - Typical grouping of basophilic and polychromatic normoblasts around a pronormoblast
B - Mitosis in an oxyphilic normoblast
C - Cell cluster containing a macrophage with phagocytosed nuclei and plastids
D - Ring of normoblasts around a central space (containing poorly stained pronormoblasts ?)

12 DAY LIVER SMEARS

FIGURE 21.

13 day embryo: Groups of cells of the erythropoietic series were characteristic once more. As in previous days, the sizes of these groups varied, and multiple numbers of each stage were present. Polychromatic and basophilic normoblasts appeared to be the most numerous, but smears of this day showed more oxyphilic normoblasts and erythrocytes.

One new feature was observed. In one smear, a particularly large colony was present (almost suggesting a sheet of cells peeled off the surface of the liver), in which were scattered occasional large megakaryocytes. The cytoplasmic margin of some of these megakaryocytes showed many bubbles, some of these on distinct pedicles. The appearance of this bubbling was similar in every way to the bubbling of megakaryocytes observed in the sectioned liver of older embryos.

Very large phagocytes, containing multiple pycnotic nuclei and plastids of varying size in their cytoplasm, were also observed. Very often such a phagocyte was surrounded by a ring of oxyphilic normoblasts with eccentric, pycnotic nuclei, the nuclei being closely orientated to the margin of the phagocyte.

Numerous examples of protruding pycnotic nuclei, and of pycnotic nuclei lying in close association with erythrocytes were observed in these smears.

Examples of oxyphilic cells containing numerous, densely basophilic, round particles of varying size, were also seen.

14 day embryo: The groups of cells of the erythropoietic series were similar to those found in the 13 day embryo. Smears of this stage of embryonic liver, however, showed many erythrocytes containing a fine, basophilic stippling irregularly scattered throughout the cytoplasm.

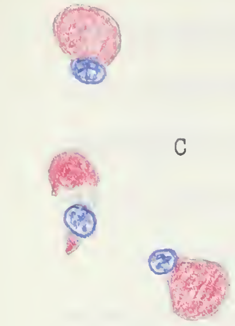
Polychromatophilic plastids with closely associated pycnotic nuclei were also observed fairly frequently.

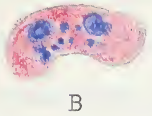
Several pronormoblasts showed bubbling of cytoplasm, similar to that seen in megakaryocyte cytoplasm.

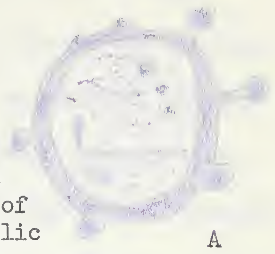
15 day embryo: The cell clusters of this day showed no new features. Once more, megakaryocytes, phagocytes, and bare pycnotic nuclei were additional features noted in the familiar groups of cells of the erythropoietic series.

B. Developmental picture over the period observed in smears:

The characteristic picture for all days studied was grouping of the cells of the erythropoietic series, each group representing a complete developmental range. (In the smears of the earliest day, the 9 day embryo, the most mature cells -- the oxyphilic normoblast and the erythrocyte -- were not always present.) In these groups of cells, one cell type was usually present in greater numbers: in the early







in a
colony of
basophilic
cells

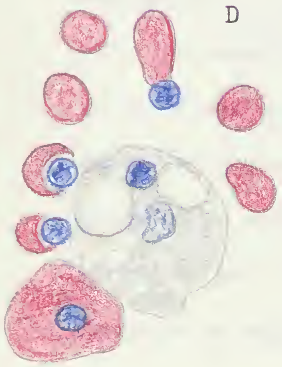
A - Megakaryocyte (20 microns in diameter) showing bubbling of cytoplasm

B - Nuclear fragmentation - fragments of varying size

C - Nuclear extrusion suggested

D - Oxyphilic normoblasts and erythrocytes closely associated with a macrophage

E - Macrophage containing pycnotic nuclei, nuclear fragments, and plastids of varying size



13 DAY LIVER SMEARS

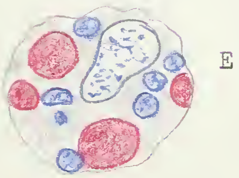


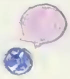
FIGURE 22.

part of the period studied, this predominant cell was the basophilic normoblast; in the later part of the period studied, the predominant cell was the polychromatic normoblast. It was also noted that for this later period, although mitoses were observed in all cell types, they seemed to occur more frequently in the polychromatic normoblast.

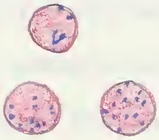
In the later part of this period, megakaryocytes and phagocytes appeared, and the occurrence of extruded nuclei increased.

C. Comparison of results of the two methods, smear and daub:

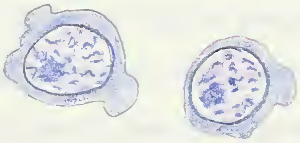
Comparison of these two methods showed the smear to be the better one. It seemed that more cells were peeled off the cut surface of the liver and deposited on the slide. Similarly, smears produced on dry slides and air-dried were superior on the whole to smears produced on methyl alcohol drained slides; once again, the cells seemed to peel off more effectively on the dry surface. Smears produced on normal-saline-drained slides gave very poor results.



- loss of nucleus from polychromatic normoblast, observed occasionally -



- basophilic stippling in erythrocytes observed frequently -



- occasional examples of pronormoblasts with pseudopodia

14 DAY LIVER SMEARS

FIGURE 23.

DISCUSSION

A survey of the results obtained from this study shows that hemopoiesis was intensive throughout the period studied, and therefore, it can be said of any one day that blood cell proliferation was marked. One fact that did show up from the differential counts, however, was that on different days, different blood cell types were most numerous. Thus, in the 10 day embryo, basophilic normoblasts constituted approximately 33% of the cells found in the liver sinusoids; in the 12 day embryo, oxyphilic normoblasts constituted 73 to 90% of the blood cell population in this area; and in the 14 day embryo, the erythrocyte constituted 61 to 73% of the cells in the sinusoids. These figures can be interpreted in several ways.

It is possible that the erythrocyte population of the 14 day embryo was the matured basophilic normoblast population of the 10 day embryo, which would mean that cell proliferation or multiplication takes place at an early stage in the developmental series. This is the viewpoint of Doan, Cunningham, and Sabin (1925) and of Undritz (1952). To connect these two cell populations in such a manner would mean that the process of maturation is a slow one, three to four days in this case. This possibility is borne out by the observation that although erythropoiesis was intensive in embryonic mouse liver from 10 days on, the first appearance of erythrocytes in the liver sinusoids in the differential counts was on the 13th day (one 12 day embryo, further advanced in development, also had erythrocytes present). Also, in the embryonic circulation, basophilic normoblasts were present in the 9 day embryo, but circulating erythrocytes were not noted in differential counts until the

13th day.

One difficulty arises in this argument. A quick comparison of the 10 day liver and the 14 day liver shows an obvious difference in the actual number of blood cells present. Thus, the 10 day liver was a relatively loose, open structure, while the 14 day liver was densely cellular, the sinusoids being packed with cells. The basophilic normoblasts must have continued to proliferate at a great rate to produce the masses of erythrocytes found at 14 days. This possibility is given some weight by our observation of many mitoses in the normoblast population of the 10 day embryo and our observation of no mitoses in the predominantly erythrocyte population of the 14 day embryo. The presence of a small number of early cells (pronormoblasts and basophilic normoblasts) actively dividing would have maintained a maturing population and would explain why a good range of developmental stages was present at any one time in the liver sinusoids.

Rather than stating that proliferation takes place at any one level, it is probably more true that proliferation takes place at all levels of development of the nucleated cell, and that a differential rate of proliferation exits, - i.e. that cell proliferation is more characteristic of some stages of development than of others. Thus, we could place the highest rate of proliferation at the level of the basophilic normoblast, but the occurrence of proliferation at succeeding levels would help explain the great increase in the actual number of cells in later days. The occurrence of mitoses at all levels down to the oxyphilic normoblast in both sections and smears substantiates this. The statements by Plum (1947) and Undritz (1952) also support this theory.

Maximow (Bloom, 1938) observed that hemocytoblasts and erythroblasts occurred in even-numbered groups, which would seem to indicate mitotic proliferation. Sections of the extrasinusoidal tissue of 11 day liver and smears of the cut surface of liver of 11 to 14 days showed that in groups of developing red blood cells, one cell type was usually present in greater numbers, and usually in even numbers. In the 11 day liver, the basophilic normoblast was the predominant cell, while the smear groupings showed the basophilic normoblast to be the main cell in the early days of the series, and the polychromatic normoblast to be the main cell later in the series. The importance of the polychromatic normoblast in these later smears poses another question.

Presuming a differential rate of proliferation, does the level of greatest proliferation change during the development of the embryo? This could be the other explanation for the difference in the differential counts of the 10 day and the 14 day embryo. Observation of the liver smears would seem to indicate a shift in proliferative level from the basophilic normoblast in the early days of erythropoiesis to the polychromatic normoblast in the later stages. The facts that the cell in question was present in greater numbers in the cell clusters, and that mitotic figures were observed more often in this cell type would seem to indicate the truth of such an assumption. Whether or not the relatively small size (5 microns) of the polychromatic normoblast in the 13 day embryo, which strangely interrupted the gradual reduction in size from the 9 by 8 micron pronormoblast to the 6 micron erythrocyte, is another indication of its great proliferative activity, depleting its available store of cytoplasm through repeated cell division, is an interesting speculation.

The proliferative level might possibly be one of the factors implicated in the failure of cultures to show division and maturation of cells of the erythropoietic series. Most of the cultures were of 12 and 13 day mouse embryonic liver, so that the majority of the blood cells migrating out into the explant would most probably have been oxyphilic normoblasts, the cell type most numerous in liver sections of those days. If mitotic division is most characteristic of basophilic or polychromatic normoblasts, and not characteristic of oxyphilic normoblasts, mitotic division would probably not be a striking feature in such cultures. Therefore, in order to obtain proliferation, cultures of earlier embryos would have to be made. Red blood cell formation has been observed in tissue cultures by such investigators as Shipley (1916) and Benewolenskaja (1929), and it is noteworthy that,in their experience, red blood cell formation was observed from amoeboid colorless elements (Shipley) and hemocytoblasts (Benewolenskaja) - in other words, from cells very early in the developmental series. Culturing embryonic liver at an earlier age, however, poses the very real difficulties of detecting and dissecting the liver.

The study of stained embryonic liver sections showed that cells of the erythropoietic series were to be found, not only in the liver sinusoids, but also in the extrasinusoidal tissue. Here, too, there was a positive increase in the number of blood cells found with growth of the liver, the extrasinusoidal tissue of the 15 day liver being packed with such cells. A very interesting contrast showed up between the developmental picture of the two areas. Whereas the blood cell population of the sinusoids had gradually shifted to a more mature cell type, the blood cell population of the extrasinusoidal tissue remained relatively immature. In the early days

of the 7 day period studied, there was a slight change from a preponderance of pronormoblasts to a preponderance of basophilic normoblasts, while throughout the rest of the period studied, basophilic normoblasts were more numerous. The occurrence of basophilic normoblasts in clusters, and the observation of mitoses in these clusters (often more numerous than in the sinusoids) would indicate that blood cell proliferation is marked in the extrasinusoidal tissue. Since oxyphilic normoblasts were very rarely encountered in the extrasinusoidal tissue it seems possible that the cells being produced here entered the sinusoids to complete their maturation, before being released into the general circulation. If entry into the sinusoid was through gaps in the endothelial lining as stated by Jordan (1942) and Pease (1956) such gaps were not observed, unless the difficulty encountered in outlining extensive portions of endothelial lining provided indirect evidence for such gaps.

It is interesting to speculate which cell type migrated into the sinusoids. Since the sinusoids usually contained a complete developmental range, any or all of the early cell types could have migrated through the membrane. An interesting possibility is that the polychromatic normoblast, which was observed on several occasions to have highly irregular cytoplasm suggestive of ameboid movement, was the cell type which switched environmental sites.

If such a continuous mass migration of early cells of the erythropoietic series into the sinusoids did take place, it is interesting to speculate about the reasons for such a migration. It seems possible that a real difference exists in the two environments and that the young blood cell enters the sinusoid to complete its maturation. Its maturation, as we know, consists in the gradual accumulation of hemoglobin in its cytoplasm

and the eventual loss of its pycnotic nucleus. Thorell (1947, 1953) has shown by cytochemical studies that two distinct, consecutive phases can be demonstrated in the life of the developing red blood cell. The first phase, which he terms the growth phase, consists in the elaboration of basic proteins (probably including the globin part of hemoglobin) in the cytoplasm of the cell; this phase is correlated with basophilia of the cytoplasm, and the presence of definite nucleoli in the nucleus. The second phase, which he terms the differentiation phase, starts after the activity of the first phase has ended (i.e. there is no overlap between the two phases). During this phase the iron-containing heme group is probably formed and the synthesis of hemoglobin occurs; this activity is correlated with increasing eosinophilia of the cytoplasm of the cell. It seems possible, then, that this growth phase takes place in the extra-sinusoidal tissue, while the differentiation phase takes place inside the sinusoid. What special property of the intrasinusoidal environment facilitates differentiation is not clear from morphological studies. Is the enclosing endothelial membrane itself the important factor, or is it the circulating fluid within that membrane, containing as it does dissolved nutritive, mineral, and hormonal substances, which is the important factor in producing a sort of chemotaxis of the young red blood cell?

The possible significance of this question is seen when we consider the problem of the failure of liver tissue cultures to demonstrate complete maturation of red blood cells. Why should a blood cell, which we recognize as started along its path of maturation by the yellowish color of its cytoplasm in tissue culture preparations, fail to reach its ultimate goal

of maturation, the loss of its nucleus. Observation of living cultures indicated that some of these blood cells increased their charge of hemoglobin, yet their numbers were relatively few considering the infrequent appearance of intensely oxyphilic normoblasts in fixed and stained culture preparations. If the assumption that a special environment is necessary for the complete differentiation of the normoblast is valid, it is evident from the failure of the cultures that this special environment was lacking. If this special environment should be intrasinusoidal, it is possible that until endothelial channels are formed more extensively in tissue cultures to harbor the maturing normoblast, differentiation will not be observed. Also, if the lacking environmental factor should be a substance in the circulating fluid of the sinusoid, although we may feel that in culturing blocks of liver tissue some of this fluid will be transferred to the culture medium, this substance may not be present in sufficient quantities or strength to provide the necessary differentiating environment. It is also possible that the negative effects of a wrong environment, provided by the chick plasma and chick embryo extract, were factors limiting the possibilities for differentiation in vitro.

Certainly, it seems from observation of the tissue cultures that growth occurred, if only in the direction of epithelium, fibroblasts, and random endothelial growth. In some cultures there was a suggestion of formation of endothelial channels, and it may be that these cultures if maintained longer, might have produced more encouraging results.

Although the actual mode of loss of the nucleus was not seen, suggestive activity on the part of normoblasts was observed. This consisted of repeated bulging in and out of the normoblast nucleus, suggesting

imminent extrusion. Post facto evidence for nuclear extrusion was observed in fixed and stained cultures: e.g. pycnotic nuclei (with a thin rim of ragged cytoplasm) in association with erythrocytes; oxyphilic normoblasts with eccentrically placed pycnotic nuclei; and normoblasts with protruding nuclei. Similar suggestive pictures were encountered in the stained sections of 13 day to 15 day liver and in the stained smears.

On the other hand, many of the configurations encountered in sectioned material, particularly of the 13th day, suggested that the pycnotic nucleus had shrunk (as evidenced by smaller size, and the appearance of a thin, surrounding halo in many polychromatic and oxyphilic normoblasts) and fallen out, leaving a very evident spherical depression in the newly formed erythrocyte. The appearance of many apparently naked nuclei in both sections and smears would appear to fit in with this observation. Whether this appearance could be due to distortion following fixation is not clear.

Suggestive evidence for both karyorrhexis and karyolysis was also observed. Basophilic fragments of varying size were often encountered, either in the spherical depression of the newly formed erythrocyte once occupied by the nucleus or in close association with this depression. In other cases, such fragments were scattered widely throughout the cytoplasm of the cell. The possibility that scattered fragments might represent chromosomes which had failed to become incorporated in the post-mitotic nucleus, as claimed by Isaacs (1938), may be borne out by the observation on liver smears that mitotic figures in oxyphilic normoblasts often appear irregular and unstructured.

The occurrence of ghost nuclei, suggesting karyolysis, was observed only rarely in either stained sections or smears, and, therefore, did not seem impressive evidence for the fate of the nucleus.

A possible ultimate fate for the nucleus after its loss by the erythrocyte was seen in the relatively common appearance in sections and smears from older embryos, of phagocytosed pycnotic nuclei. An interesting situation appeared several times in smears of 12 and 13 day embryos, where groups of oxyphilic normoblasts were arranged around the periphery of a large macrophage, their dense, pycnotic nuclei most closely orientated to its margins. It seems possible that macrophages may form a functional part of a hemopoietic cell group, removing nuclei and nuclear debris as they are lost from erythrocytes.

From observations of living cultures, fixed and stained cultures, sections, and smears, it appears that the pycnotic nucleus is lost either by the active mode of extrusion, or, that, in some cases, it does not require such active expulsion, but "falls out", as the more passive mode of loss has been termed in this discussion. It is probable that the loose nuclei are then phagocytosed by neighboring macrophages. With such numerous examples of loose nuclei in fixed material, it is difficult to fit in the observations of Muir and Kerr (1958) of the gradual disappearance of the nucleo-cytoplasmic boundary due to the formation of hemoglobin in both cytoplasm and nucleoplasm.

The related question of the fate of the erythrocyte itself arises. Observations of living tissue cultures did not throw any light on this question, but in the stained cultures, sections, and smears, there were occasional examples of macrophages which contained not only pycnotic normoblastnuclei, but hemoglobin-containing plastids of varying size. These macrophages were especially conspicuous in liver smears of 12 and 13 day embryos. It could be emphasized once more that they often appeared to form an integral part of the cell clusters so characteristic of these smears,

this appearance being most marked where a ring of oxyphilic normoblasts and erythrocytes were clustered around a macrophage already containing phagocytosed nuclei and plastids.

On the other hand, it was quite common to encounter in stained liver sections a rather indefinite debris in large sinusoids of the liver which might indicate cellular disintegration. It is possible that such an appearance could be due to the trauma of fixation.

It has been stated that the macrophages contained plastids of varying size. This observation would fit in with the statement by Doan and Sabin that the erythrocyte becomes fragmented, and the fragments then become phagocytosed. The process of erythrocyte fragmentation is described in detail by Doan and Sabin (1926) and is corroborated in the observations of Cooley and Lee (1933). The red cell puts out a long process which begins to vibrate slowly, then progressively faster, while at the same time its connection with the main cell is becoming thinner until the process ultimately separates and a fragment of varying size is formed. In liver tissue cultures, it was observed that the erythrocyte displayed on occasion a continuing change of shape, psuedopodia being protruded and withdrawn. It seems possible that this phenomenon could very well have been the initial stages of erythrocyte fragmentation, and that the fragmentation process, like other blood cell activities in vitro, was only abortively performed, the blood cell failing in vitro to complete activities performed in vivo.

The descriptions of cytoplasmic constriction by Emmel (1914) and of gemmation or protoplasmic segregation by Plum (1947), producing plastids of varying size and shape, are perhaps suggestive of the process of fragmentation described above. However, both Emmel and Plum have described the formation of these plastids from nucleated normoblasts whereas

fragmentation occurs in the non-nucleated erythrocyte.

Observations of stained liver sections show a considerable range in the diameters of erythrocytes (8 microns to 2 microns). Whether the small erythrocytes represent fragmented forms or gemmation forms budded off parent normoblasts is not clear from the study of sections or smears. It is also possible that the extremes of this range constitute representatives of two separate series of red blood cells, a possibility which will be discussed below.

The range in size of the erythrocyte in sectioned material has been commented on. A survey of cell measurements taken over the seven day period shows a considerable variation in size for each cell type. This variation is present in all days studied. For several of the cells (pronormoblast and oxyphilic normoblast) the range of cell measurements was greatest in the early stage (9 day embryo), but this observation did not hold true for all cell stages. Cell measurement in this study was intended to show average measurements of cells encountered and a comprehensive systematic survey of the range of cell sizes found in the liver and in the circulation was not carried out. A survey was carried out in a limited manner on the cells of the liver and circulation in the 13 day embryo, and the results of these measurements showed close comparison of cell sizes in these two areas.

Since cell measurement was one of the criteria involved in distinguishing cell members of the transient,primitive red blood cell series (the megaloblast series) from members of the permanent,definitive red blood cell series, it might well be that the larger oxyphilic normoblasts and erythrocytes were members of the primitive series and that the smaller

cells were members of the definitive series. An additional criterion was the presence of a more delicate chromatin network in the nuclei of cells of the primitive series. A survey of the normoblasts encountered in this study would certainly show occasional examples of such nuclei, and might constitute further evidence for the existence of these two series in the material studied. Since it is well accepted that the primitive series issues from the yolk sac vessels and such vessels were present in the early days of the material studied (under the terminology of fetal membrane vessels), it is entirely possible that members of this series might have been present in the circulation of the embryos studied. Since many of the measurements quoted, however, were of cells encountered in the liver sinusoids, it is not clear whether these large primitive cells (presuming they were of the primitive series) were formed in the liver, or reached the liver through the general circulation. The possibility of primitive series cells being formed in the liver would introduce a controversial issue since it is generally well accepted that the embryonic liver gives rise to the definitive or normoblast series only. It is obvious that the observations recorded in this study were not orientated in the direction of detecting the presence of members of these two series as were such investigations as those of Jones (1934) and Kirschbaum (1937) which dealt in great detail with the differentiation of the two series, and it would be a mistake to attach too great importance to random observations on the question in this study. Indeed, to have followed up this very interesting question would have constituted a definite distraction from the main purpose of this investigation.

One of the maturation changes in the normoblast which was followed

with a degree of success in tissue cultures of embryonic liver was the accumulation of hemoglobin in the cytoplasm of the cell.

The yellowish color due to the presence of hemoglobin was observed to increase in intensity although whether the cell developed a full complement of hemoglobin in vitro is not clear. It seems probable that a failure to develop a full content of hemoglobin could be correlated with a failure to reach full maturation status, as evidenced by nuclear loss. It is interesting to note that although Undritz (1952) commented on the sudden increase in hemoglobin in a cell during mitosis, blood cells in tissue cultures of this study showed increase in hemoglobin content without mitosis.

While a relationship between mitosis and hemoglobin accumulation has not been observed in these tissue cultures, the observation of Emmel (1914) that the nucleus tends to assume an eccentric position in the cell (due to the accumulation of hemoglobin at one pole displacing the nucleus to the opposite pole) is substantiated. In the fixed and stained cultures of 12 day liver, many examples occur of a dense nucleus at one pole, and a globular mass of hemoglobin at the opposite pole.

Emmel's (1914) conclusion that the assumption of the definitive form (flattened biconcave disc or concavo-convex cup) is not necessarily dependent upon the extrusion of the nucleus receives support in the observations of oxyphilic normoblasts in 13 day liver sections. Here, some oxyphilic normoblasts exhibit biconcave or concavo-convex forms with the nucleus still present, while other erythrocytes show biconcave and concavo-convex forms coincident with a spherical depression, obviously recently occupied by the nucleus.

No inner structure was observed in the cytoplasm of erythrocytes

in living cultures or in fixed and stained preparations. This is in keeping with the electron microscopic findings of Rebuck (1948) and Bessis (1950). The statement of Isaacs (1938) that the living cell is soft and easily molded by surrounding structures would explain the numerous irregular forms of erythrocytes in the densely cellular sinusoids of the liver sections. Such irregular forms might also be due to the trauma of fixation. Here it is worthwhile noting that Buchsbaum (1947) checked the validity of several histological procedures using phase microscopy and found that Zenker-formol and Maximow's Zenker-formol were the fixatives that yielded images most like the living cell. He also noted that the histological procedures following fixation produced very little further distortion of the cell. It seems safe to presume, therefore, that the irregular forms of erythrocyte encountered in fixed material exhibited these irregularities in vivo and were fixed while possessing this form.

The only evidence available in this study on the life span of the erythrocyte is the appearance of phagocytosed erythrocyte fragments in stained sections and smears of 12 to 15 day embryonic liver. Since this is coincident with the first appearance of erythrocytes in the embryo (barring atypical larger embryos showing further development), it is not safe to assume that these phagocytosed erythrocytes had pursued a normal life span. Also, since most studies such as that of Wearn, Warren and Ames (1922) on the life span of the erythrocyte, have been done on adult patients (often anemic patients) it is not safe to assume, firstly, that the life span is identical in healthy and anemic subjects, and secondly, that the life span is identical in adult and embryonic tissues.

It was hoped, in observing tissue cultures of embryonic mouse liver, to study the actual movements of cells. This expectation was fulfilled in

a very limited way only. In actual fact, the movements of cells in tissue culture were extremely difficult to appreciate. The actual change in form was so gradual and so extremely slow that in many instances the change was completed before the process of change could be recognized. Practiced observation was required and even with the most intensive visual application it was difficult to appreciate that an infinitesimal change in shape in one area of the cell might represent a major protrusion minutes later, especially if a very evident pseudopodic process at another place was being observed simultaneously! It was soon evident that the task of observing and recording change in form must necessarily be performed by a more impartial eye, and this is one area in tissue culture research where the aid of cinematographic timed records will be invaluable. Movements of an erythrocyte during a 15 minute observation period are portrayed in Fig. 18. These have been interpreted as possible pre-fragmentation movements, in line with the descriptions given by Doan and Sabin (1926).

It is obvious that movement of blood cells must occur if we are to explain the migration of blood cells in tissue cultures out of the explant into the area of outgrowth. Since it was blood cells of the erythropoietic series which were observed to exhibit this phenomenon in the cultures studied, this is a direct contradiction of the statement by De Bruyn (1944) that cells of the red blood cell series do not show independent active locomotion.

The study of stained liver smears and sections has produced other observations not directly connected with the aims of this investigation, but, since they are of interest in the study of hemopoiesis, they will be discussed briefly below.

In answer to the question posed earlier, "Where is blood formed?", the material studied showed that hemopoiesis occurred not only in the liver, but also in the vessels of the embryo and of the embryonic membranes, especially in the early part of the period studied. One question might be raised here. Were the basophilic normoblasts which were observed in early embryonic vessels circulating blood cells or did they merely represent blood cells formed in situ? It is also interesting to ask why basophilic blood cells should circulate at all if they have not yet attained their definitive oxygen-carrying ability. It seems more probable that the basophilic blood cells observed in embryonic blood vessels were formed there. The occurrence of many mitoses in these cells would seem to substantiate this possibility. Basophilic and polychromatic normoblasts, occurring singly or in small groups, were also observed in unlined spaces in the body mesenchyme of 9, 10, and 13 day embryos, which would support the observations of Maximow (1924).

The question of sites of blood cell formation is intimately related to the question, "What cells produce blood cells?" The assumption that the basophilic blood cells observed in embryonic blood vessels were formed in situ rests on the previous assumption that the endothelium of such vessels can give origin to cells of the erythropoietic series. Mitoses in endothelial cells, endothelial cells exhibiting basophilia of cytoplasm, and endothelial cells bulging into the lumen of vessels, were all observed in embryos up to 13 days of age, and were more numerous in the youngest embryos. In one 10 day embryo section the lining of the left atrium showed many basophilic endothelial cells bulging into the heart chamber, and basophilic normoblasts free in the lumen. On the basis of this admittedly

indirect evidence, it seems entirely probable that at least in young embryos, endothelial cells are capable of giving origin to cells of the erythropoietic series. The occurrence of early cells of the erythropoietic series in unlined mesenchymal tissues would indicate that embryonic mesenchymal cells also exhibit an erythropoietic activity.

On the basis of this limited study, it is not possible to take a very firm stand in the monophyletic-polyphyletic controversy. Cells of the granular white blood cell series were not observed in the age range studied, and it was only in 14 and 15 day embryos that the appearance of a lymphocyte-like cell was observed. It is possible to state that in the extrasinusoidal tissue of the liver where this new cell type was observed, no large, young cell was encountered other than the familiar pronormoblast. The possibility that the cell termed a lymphocyte in this study was derived from the same stem cell which produced the normoblast series, would seem to constitute indirect evidence for the monophyletic theory.

The following observations were made on the occurrence of megakaryocytes. These huge cells first appeared in the 12 day embryo and throughout the rest of the period studied they were observed to occur in the extrasinusoidal tissue of the liver, characteristically in a clear zone immediately bordering a sinusoid. Indeed, it became almost axiomatic that the area in which to search for these cells was the immediate vicinity of the large liver sinusoids. Whether this intimate relationship of megakaryocytes to sinusoids was related to their ultimate budding of platelets into the circulation can only be conjectured. 13 day smears and 14 day sections of liver showed a curious bubbling of the megakaryocyte cytoplasm. This appearance of bubbling might have been construed to indicate a distortion of the cell resulting

from the trauma of being pulled across the glass slide, had it not been for the appearance of an identical picture in sections of the liver of 14 days and thereafter. The occurrence of small, globular bodies of the same general size range as platelets, in the immediate vicinity of these megakaryocytes, led to the assumption that the bodies were platelets budded off the cytoplasm of the megakaryocytes. This assumption seems reasonable since these so called platelets were the same size as, and exhibited the identical staining properties of the cytoplasmic bubbles and,more important, they did not appear in sections until after the appearance of megakaryocytes. It also seems probable that the megakaryocyte went through a maturation period since the cytoplasm of later cells exhibited more polychromatophilia, and since the appearance of bubbling was not observed coincident with the first occurrence of megakaryocytes. It is difficult to comment on the origin of the megakaryocyte except to remark that its association in the extrasinusoidal tissue with early cells of the erythropoietic series might indicate a possible origin from the same stem cell.

On the question of granules in the near-mature members of the erythropoietic series, it is only possible to state that these occurred. Examples of what appeared to be basophilic stippling were observed in 14 day liver smears; apparent examples of substantia granulofilamentosa were noted in 15 day liver sections; and numerous pictures which might be described as basophilic granules, and nuclear particles were encountered in the older embryos. Exact differentiation of these particles would require appropriate specific staining, a project apart from the central problem of this study.

SUMMARY

1. General studies on erythropoiesis were made of mouse embryos from the age when the liver first appeared to the 15th day.
2. The embryonic ages at which different cells of the erythropoietic series first appeared were established.
3. The embryonic age at which nuclear loss from normoblasts first occurred was recorded.
4. Liver tissue cultures of mouse embryos ranging in age from 11 to 15 days failed to demonstrate proliferation or full maturation of cells of the erythropoietic series.
5. The actual mode of loss of the normoblast nucleus was not observed in these tissue cultures.
6. Incidental observations on hemopoiesis were made from the study of sections, cultures, and smears.

REFERENCES

1. ASHBY, W. 1948. The Span of Life of the Red Blood Cell. Blood 3: 486-500.

2. BENEWOLENSKAJA, S.W. 1929. Hematopoiesis in cultures of the Embryonic liver in man. Arch. exp. Zellforsch 9: 128-141.

3. BESSIS, M. 1950. Studies in Electron Microscopy of Blood Cells. Blood 5 (12): 1083.-1098.

4. BESSIS, M. 1955. Phase Contrast Microscopy and Electron Microscopy Applied to the Blood Cells. Blood 10 (3): 272-286.

5. BLOOM, W. 1938. The Embryogenesis of Mammalian Blood in Downey's Handbook of Hematology 2: 863-922, New York, Hoeber, 1938.

6. ________________ Tissue Cultures of Blood and Blood-forming Tissues in Downey's Handbook of Hematology 2: 1469-1549, New York, Hoeber, 1938.

7. BLOOM, W., and BARTELMEZ, G.W. 1940. Hematopoiesis in young human embryos. Am. J. Anat. 67: 21-53.

8. BUCHSBAUM, R. 1947. Individual cells observed under phase microscopy, during fixation. Anat. Rec. 99: 640.

9. COOLEY, T.B. and LEE, P. 1933. The Role of Erythrocyte Fragmentation in the Genesis of Anemia. J. Pediat. 3: 55-63.

10. CUPP, C.D. 1915. On the structure of the erythrocyte. Anat. Rec. 9: 259-280.

11. DANCHAKOFF, V. 1916. Concerning the conception of potentialities in the embryonic cells. Anat. Rec. 10: 415-416.

12. DE BRUYN, P.P.H. 1944. Locomotion of blood cells in tissue cultures. Anat. Rec. 89: 43-63.

13. DOAN, C.A., CUNNINGHAM, R.S., and SABIN, F.R. 1925. Experimental studies on the origin and maturation of avian and mammalian red blood cells. Carnegie Institute of Washington, Contributions to Embryology 78: 163-226.

14. DOAN, C.A. and SABIN, F.R. 1926. Normal and pathological fragmentation of red blood cells: the phagocytosis of these fragments by desquamated endothelial cells of the blood stream; the correlation of the peroxidase reaction with phagocytosis in mononuclear cells. J. Exp. M. 43: 839-850.

15. EMMEL, V.E. 1914. Concerning certain cytological characteristics of the erythroblasts in the pig embryo and the origin of non-nucleated erythrocytes by a process of cytoplasmic constriction. Am. J. Anat. 16: 127-205.

16. __________ 1916. The cell clusters in the dorsal aorta of mammalian embryos. Am. J. Anat. 19 (3): 401-421.

17. __________ 1924. Studies on the non-nucleated elements of the blood. Am. J. Anat. 33: 347-405.

18. GRUNEBERG, H. 1943. The Development of Some External Features in Mouse Embryos. Jour. Heredity 34(3): 89-92.

19. HALL, B.E. 1938. Evaluation of the Supravital Staining Method in Downey's Handbook of Hematology 1: 641-698, New York, Hoeber, 1938.

20. HAM, A.W. 1957. Histology, ed. 3, Montreal, Lippincott, 1957.

21. ISAACS, R. 1937. Formation and Destruction of Red Blood Cells. Physiol. Rev. 17(2): 291-303.

22. __________ 1938. The Erythrocytes:in Downey's Handbook of Hematology 1: 1-157, New York, Hoeber, 1938.

23. JONES, O.P. 1934. The origin of megaloblasts and normoblasts in biopsied human marrow and the difference between the two series. Anat. Rec. 58: Supplement, p. 23.

24. ________________ The reticulo-endothelial origin of erythrocytes in biopsied human marrow. Anat. Rec. 61: Supplement, p. 28-29.

25. __________ 1948. Nuclear Structure versus Nuclear Pattern. Blood 3: 967. -986.

26. JONES, O.P. and SMITH, A. 1950. Transmission of Antianemic Principle across the Placenta and Its Influence on Embryonic Erythropoiesis. 11. Comparison of the Effect of Liver Extract and Pteroylglutamic Acid (PGA). Blood 5: 499-521.

27. JORDAN, H.E. 1916. Evidence of hemogenic capacity of endothelium. Anat. Rec. 10: 417-420.

28. __________ 1938. Comparative Hematology:in Downey's Handbook of Hematology 2: 699-862, New York, Hoeber, 1938.

29. __________ 1942. Extramedullary Blood Production. Physiol. Rev. 22: 375-384.

30. KIRSCHBAUM, A. 1937. Cytological Studies of Mammalian Embryonic Blood Cells. Proc. Soc. Exp. Biol. 35: 542-544.

31. KIRSCHBAUM, A. 1937. The red blood cells of mammalian embryos. Anat. Rec. 67: Supplement (11) 67, p. 30.

32. KIRSCHBAUM, A. and DOWNEY, H. 1937. A comparison of some of the methods used in studies of hemopoietic tissues. Anat. Rec. 68: 227.

33. LEE, Bolles 1950. The Microtomist's Vade-Mecum, ed. 11, Philadelphia, The Blakiston Company, 1950.

* see 63

34. MANN, F.C., SHEARD, C.H., BOLLMAN, J.L., and BALDES, E.J. 1925. The Site of the Formation of Bilirubin. Am. J. Physiol. 74: 497-510.

35. MAXIMOW, A.A. 1924. Relation of Blood Cells to Connective Tissues and Endothelium. Physiol. Rev. 4: 533-563.

36. ______________ 1925. Tissue cultures of young mammalian embryos. Carnegie Institute of Washington, Contributions to Embryology 78: 47-114.

37. MAXIMOW, A.A. and BLOOM, W. 1957. A Textbook of Histology, ed. 7, Philadelphia, Saunders, 1957.

38. MORRIS, R.S. 1909. Nuclear Particles in the Erythrocytes. Arch. Med. Int. 3: 93-97.

39. MORRIS, R.S. and THAYER, W.S. 1911. Ameboid Movements in Macrocytes and Megaloblasts. Arch. Med. Int. 8: 581-590.

40. MUIR, A.R. and KERR, D.N.S. 1958. Erythropoiesis: An Electron Microscopical Study. Q.J. Exp. Physiol. 43(1): 106-114.

41. MURRAY, P.D.F. 1932. The Development in vitro of the Blood of the early Chick Embryo. Proc. R. Soc., Ser. B, Biol. Sc., 111: 497-521.

42. OSOGOE, B. and ŌMURA, K. 1950. Transplantation of hematopoietic tissues into the circulating blood. 11. Injection of bone marrow into normal rabbits, with special reference to the histogenesis of extra-meduallary foci of hematopoiesis. Anat. Rec. 108: 663-685.

43. PEASE, O.C. 1956. An Electron Microscopic Study of Red Bone Marrow. Blood 11(6): 501-526.

44. PLUM, C.M. 1947. In Vitro Study of Bone Marrow. 11. Studies of Erythropoiesis. Blood, Special Issue No. 1: 42-53.

45. REBUCK, J.W. and WOODS, H.L. 1948. Electron Microscope Studies of Blood Cells. Blood 3: 175-191.

46. ROSENTHAL, N. 1938. Blood Platetlets and Megacaryocytes: in Downey's Handbook of Hematology 1: 447-496, New York, Hoeber, 1938.

47. ROUS, P., and ROBERTSON, O.H. 1917. The normal fate of erythrocytes. 1. The findings in healthy animals. J. Exp. M. 25: 651-664.

48. SABIN, F.R. 1922. On the Origin of the Cells of the Blood. Physiol. Rev. 2: 38-69.

49. SCHWIND, J.L. 1950. The Supravital Method in the Study of the Cytology of Blood and Marrow Cells. Blood 5: 597-622.

50. SHIPLEY, P.G. 1916. The development of erythrocytes from hemoglobin-free cells and the differentiation of heart muscle fibers in tissue cultivated in plasma. Anat. Rec. 10: 347-353.

51. SNELL, G.D. 1941. Reproduction. Biology of the Laboratory Mouse, 55-88, Philadelphia, The Blakiston Company, 1941.

52. STOCKARD, C.R. 1915. The origin of blood and vascular endothelium in embryos without a circulation of the blood and in the normal embryo. Am. J. Anat. 18: 227-327.

53. ____________________ A study of Wandering Mesenchymal Cells on the Living Yolk-Sac and their developmental Products: Chromatophores, Vascular Endothelium and Blood Cells. Am. J. Anat. 18: 525-594.

54. STURGIS, C.C., ISAACS, R., and SMITH, M. 1928. The Treatment of Pernicious Anemia with a Liver Extract. Ann. Int. M. 1: 983-995.

55. THOMSON, D. 1914. Some Observations on the Development of Red Blood Cells as seen during the Growth of Embryonic Chick Tissue in vitro. Proc. R. Soc. M. 7, Part 1: 77-86. Marcus Beck Laboratory Reports - No.5.

56. THORELL, B. 1947. Studies on the Formation of Cellular Substances during Blood Cell Production. Acta med. scand. Suppl. 200: 1-117.

57. ___________ 1953. The Formation of Haemoglobin during the Development of the Erythrocytes. J. Embryol. 1(3): 235-238.

58. UNDRITZ, E. 1952. Sandoz Atlas of Haematology, Basle, Switzerland, Sandoz Ltd., 1952.

59. WAJDA, S.H. 1947. Origin of Erythrocytes. Nature 159: 254-256. Discussion of article by W.E. Le Gros Clark, Nature 159: 579.

60. WEARN, J.T., WARREN, S., and AMES, O. 1922. The Length of Life of Transfused Erythrocytes in Patients with Primary and Secondary Anemia. Arch. Med. Int. 29: 527-538.

61. WOODARD, W.C. and POMERAT, C.M. 1953. The development of patent blood vessels from adult human rib marrow in tissue culture. Anat. Rec. 117: 663-683.

62. YAMADA, E. 1955. Some features of the fine structure of megacaryocytes in the mouse spleen. Anat. Rec. 121: 458. Abstract D 42.

63. MANN, F.C. and HIGGINS, G.M. 1938. The System of Fixed Histocytes in the Liver: in Downey's Handbook of Hematology 2:1375 - New York, Hoeber, 1938.

APPENDIX

(SPECIMEN WORK SHEETS)

OBSERVATION WORK SHEET FOR SECTIONED MATERIAL

General: embryonic age
series designation
fixation and stain
average embryonic measurement
suitability of cellular detail

Embryonic Liver:

1. presence
2. degree of organization
3. hemopoietic activity
4. blood cell population

A. In Sinusoids

1. r.b.c.:-
    - a. stages present
    - b. numbers of each stage
      (see counts)
    - c. sizes of each stage
      (grid measurements)
    - d. extrusion of nuclei
        - any relation of extrusion to amount of Hbgln
        - any loose nuclei around
        - any phagocytosed nuclei
    - e. suggestions of: Karyolysis: Karyorrhexis
    - f. mitoses
        - seen in what cell stages
    - g. budding
    - h. shape of adult red -
        - any reln. to nuclear loss
        - any suggestion of cytoplasmic structure
    - i. presence of granules

2. w.b.c.:- types seen

3. megakaryoctyes:

4. platelets:

5. Endothelial activity:

B. Extrasinusoidal

1. r.b.c.:-
    - a. stages present
    - b. numbers of each stage
      (see counts)
    - c. sizes of each stage
      (grid measurements)
    - d. extrusion of nuclei
      any relation of extrusion to amount of Hbgln
      any loose nuclei around
      any phagocytosed nuclei
    - e. suggestions of: Karyolysis: Karyorrhexis
    - f. mitoses
      seen in what cell stages
    - g. budding
    - h. shape of adult red -
      any reln. to nuclear loss
      any suggestion of cytoplasmic structure
    - i. presence of granules

2. w.b.c.:- types seen

3. megakaryocytes:
    - a. numbers
    - b. size (grid measurement)
    - c. nuclear detail
    - d. mitoses
    - e. presence of granules

4. platelets:
    - a. size (grid measurement)
    - b. granules
    - c. topographical relationship to other cells.

C. Incidental Notes on observations made on:

1. fetal circulation:
    - a. endothelial activity
    - b. types of blood cells seen
    - c. blood cell mitoses

2. blood vessels in fetal membranes
    - a. endothelial activity
    - b. types of blood cells seen
    - c. blood cell mitoses

OBSERVATION WORK SHEET FOR CULTURES

General: - embryonic age
series designation
fixation and stain
average embryonic measurement
suitability of cellular detail
tissue cultured
type of culture

A. OBSERVATIONS ON LIVING CULTURE:

Age of culture:
Condition of culture:

1. type of outgrowth:

2. formation of blood channels:

3. presence of blood cells:
   types:

   numbers:

4. activity of cells:
   - a. cell movement
   - b. mitoses
   - c. budding
   - d. nuclear extrusion
   - e. cytoplasmic segregation
   - f. accumulation of hemoglobin

B. OBSERVATIONS ON FIXED AND STAINED CULTURE:

Age of culture:
Condition of culture:

1. type of outgrowth:

2. formation of blood channels:

3. presence of blood cells: types: numbers

4. evidence for: nuclear extrusion or cytoplasmic segregation:

   budding:

   proliferative stage (i.e. mitoses):

OBSERVATION WORK SHEET FOR SMEARS

A. GENERAL: embryonic age
series designation
fixation and stain
tissue smeared
suitability of cellular detail
type of smear

B. OBSERVATIONS:

1. Grouped cells: typical - not typical -

   a. number in a group -

   b. cell types in a group -

   c. numbers of each type -

   d. arrangement of cell types within group

2. Non-grouped cells:

   cell types seen:

   a. red series-

   b. white series-

   c. megakaryocyte series-

Cell Measurement & Mitoses

Age:

Series designation

Cell	Presence of Mitoses	Measurements										
		1	2	3	4	5	6	7	8	9	10	Av
1. pronormoblast												
2. basophilic normoblast												
3. polychromatic normoblast												
4. oxyphilic normoblast												
5. erythrocyte												
6. megakaryocyte												
7. platelets												

Differential Counts:- Age -

Series designation	Counts									
	1	2	3	4	5	6	7	8	9	10
A. Intrasinusoidal										
1. pronormoblast										
2. basophilic normoblast										
3. polychromatic normoblast										
4. oxyphilic normoblast										
5. erythrocyte										
6. megakaryocyte										
Total										
mitoses										
loose nuclei										
endothelial activity										
B. Extrasinusoidal										
1. pronormoblast										
2. basophilic normoblast										
3. polychromatic normoblast										
4. oxyphilic normoblast										
5. erythrocyte										
6. megakaryocytes										
Total										
mitoses										
loose nuclei										
endothelial activity										
C. Embryonic Circulation										
1. pronormoblast										
2. basophilic normoblast										
3. polychromatic normoblast										
4. oxyphilic normoblast										
5. erythrocyte										
6. megakaryocytes										
Total										
mitoses										
loose nuclei										
endothelial activity										

B29780